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ENDOCRINOLOGY

FEBRUARY, 1950

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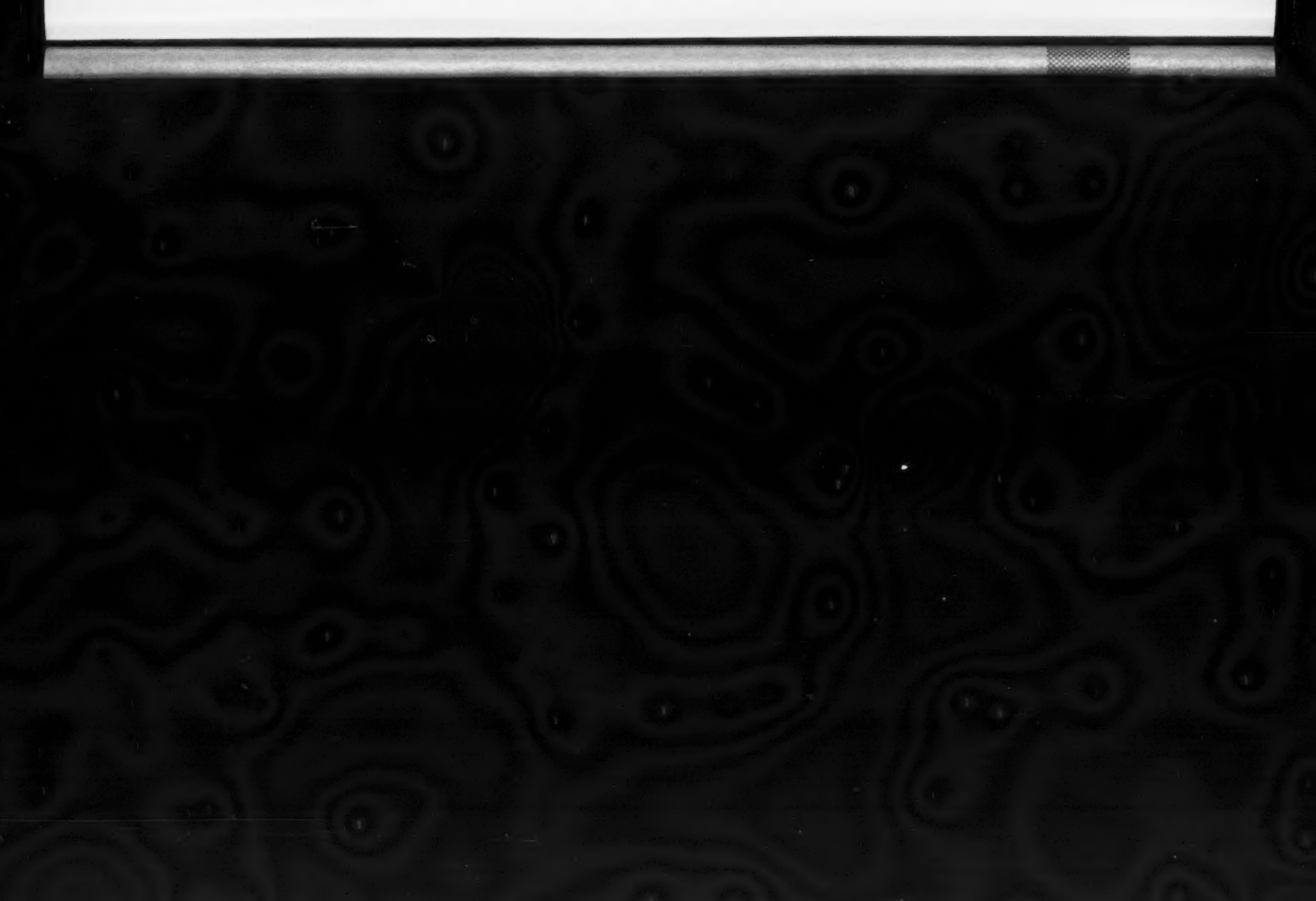

ENDOCRINOLOGY

VOLUME 46 FEBRUARY, 1950 NUMBER 2

ALLOXAN ADMINISTRATION IN THE GUINEA PIG: A STUDY OF THE HISTOLOGICAL CHANGES IN THE ISLANDS OF LANGERHANS, THE BLOOD SUGAR FLUCTUATIONS, AND CHANGES IN THE GLUCOSE TOLERANCE[1]

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SINCE the discovery in 1943 by Dunn, Sheehan and McLetchie that the injection of the drug alloxan results in specific injury to the B cells in the islands of Langerhans of the rabbit, alloxan diabetes has been produced in several species of experimental animals: rabbits, rats, dogs, cats, mice and monkeys. Although there are both species and individual differences in the degree of the response to the administration of alloxan, the qualitative effect is constant. Histologically, there is a selective necrosis of the B cells of the islets of Langerhans, with either complete disappearance of these cells (dog) or their replacement by agranular cells (rat, rabbit, monkey). In either case, there is a resultant lack of insulin-producing tissue. The A cells apparently remain unharmed by the alloxan (Goldner and Gomori, 1943, 1944 c; Hard and Carr, 1944; Duffy, 1945; Ruben and Yardumian, 1945; Duff, 1947).

Permanent alloxan diabetes has not been produced in the guinea pig. Goldner and Gomori (1944 a) found that all of their animals which showed degenerative lesions in the pancreas died within the first 24 hours. Those which survived beyond the first day showed no islet pathology. Saviano and De Franciscis (1947) found varying degrees of necrosis in the pancreatic islets accompanied by a prolonged and fatal hypoglycemia in most cases. Griffiths (1948) was able to produce an hypoglycemic reaction in normal guinea pigs by injecting

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[1] Aided by a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation.

the alloxan directly into the pancreas, and in guinea pigs maintained on a methionine and cystine deficient diet by intraperitoneal injection. Islet cell necrosis was reported only in those animals which received the intrapancreatic injection. West and Highet (1948) and Charalampous and Hegsted (1949) observed no significant blood sugar changes following various dosages of alloxan. The latter authors reported islet lesions of variable severity and death within three days with the higher dosages.

The islands of Langerhans of the guinea pig pancreas are well-defined and the cell types are easily distinguished with differential staining techniques. It seemed of interest, therefore, to study the cytological changes in the islet cells of the guinea pig following alloxan administration, to determine the effect of various dosages, the most effective route of administration, and the correlated blood sugar fluctuations.

The apparent complete recovery of the guinea pigs from the effects of alloxan within a few days after injection led to studies to determine the capacity of the normal and alloxan-treated guinea pig to utilize an excess of exogenous carbohydrate. For this purpose, several series of glucose tolerance tests were performed before and at intervals after alloxan administration. The histological appearance of the islets and the proportion of B to A cells in the normal and alloxan-treated guinea pig served as an estimate of the degree of recovery in the islet tissue itself.

MATERIALS AND METHODS

Male guinea pigs weighing between 265 and 740 grams were used. Freshly prepared 5% solutions of alloxan[2] in distilled water were injected subcutaneously, intraperitoneally or intravenously in amounts equivalent to 100, 150, 175, 189, 200 or 300 mg. per kilo of body weight. Intravenous administration was found to be the most effective, and was therefore used in the majority of the experiments. Some of the animals were fasted overnight (14 to 18 hours) prior to injection of alloxan. Those which became weak, hyperirritable or convulsive during the first 12 hours after alloxan injection were given 1 gram of glucose (10% solution) per kilo subcutaneously or intraperitoneally to allay the hyperglycemia. This was repeated as often as the physiological condition necessitated.

Blood samples were obtained either by cardiac puncture or from the ear veins. By careful nicking of the marginal ear veins with a razor blade, as described by Woerner (personal communication), and the use of very small amounts of heparin, it was possible to secure frequent samplings of 0.1 cc. each. After injection of alloxan, blood samples were taken either at 15-minute intervals during the first hour and hourly thereafter up to 8 hours, or at 2-hour intervals up to 8 hours. Whenever possible, 12, 24, 48, 72 and 96-hour samples were also obtained. Duplicate blood sugar analyses were carried out according to the iodometric method of Somogyi (1945 a, b).

For the glucose tolerance tests, the animals were fasted overnight (14 to 18 hours) and 2 grams of glucose (10%) per kilo injected intraperitoneally.

[2] Alloxan (monohydrate), Eastman Kodak Company, Rochester, N. Y.

Blood sugar determinations were made on samples taken immediately before and ½, 1, 2, 3, and 5 hours after the glucose injection.

At various intervals after alloxan injection the animals were sacrificed (by decapitation, hemopericardium or illuminating gas) and the pancreas removed. Small pieces of the tail of the pancreas were fixed in Bouin's fluid, dehydrated and embedded in paraffin. Sections cut at 4 μ were stained with chrome hematoxylin and phloxin (Gomori, 1941).

Biopsies of the pancreas were done on several of the experimental animals. Under ether anesthesia, a small piece of the pancreas was removed and prepared as above for microscopic examination. All tissues were studied with a 10× ocular and 1.8 mm. oil immersion objective.

Since the A and B cells of the guinea pig are easily distinguished with the chrome hematoxylin-phloxin stain, the proportion of the two types of islet cells could be determined in both control and experimental animals. This was done by systematically counting 1000 islet cells (nuclei) in a section and subtracting the number of A cells. This was repeated 3 times in preparations from each animal and the results averaged.

Accurate measurements of individual B cells were found to be difficult due to the obscurity of many of the cell outlines.

RESULTS

The effective dosage of alloxan

The degree of the response of guinea pigs to alloxan administration was found to vary with the route of administration, the dosage, and whether or not the animals were fasted prior to injection. Early in the work it was found that as much as 200 mg. alloxan given subcutaneously or intraperitoneally produced no effect in non-fasted animals and only a minimum response in fasted animals. Consequently, in the subsequent series of 77 animals the alloxan was given intravenously in dosages of 100, 150, 175, 189, 200 and 300 mg. per kilo.

Aside from a few animals (9%) which were found to be refractory at the dosages used, the effects varied from necrosis of a few B cells and a slight blood sugar effect (minimal response) to degeneration of nearly all the B cells and pronounced but not permanent blood sugar changes (maximal response). The number of B cells killed with the 100 mg. dosage was less than with 200 mg.; nevertheless the damage was sufficient to produce a blood sugar curve indistinguishable from that resulting from the 200 mg. dosage (Figure 1 A and Table 3). The smaller dosages (100 and 150 mg.) were ineffective in non-fasted animals whereas there seemed to be no difference between the response of fasted and non-fasted animals when 200 mg. was given (Table 1). Five animals given 300 mg. alloxan per kilo had no more islet cell damage than with the 200 mg. dosage. All animals with the high dosage died within 72 hours, however, and had extensive liver and kidney lesions. In no case was a permanent diabetes established so that the dosages producing extensive islet cell lesions or definite blood sugar changes are termed "effective" rather than diabetogenic.

Blood sugar studies

The blood glucose level of blood obtained from the heart (left ventricle) by cardiac puncture (93 ±15 mg.%) was found not to differ significantly from that obtained from the ear veins (98 ± 11 mg.%). As a result of 63 determinations on 39 different normal guinea pigs, the average fasting blood sugar was found to be 95 mg. per cent

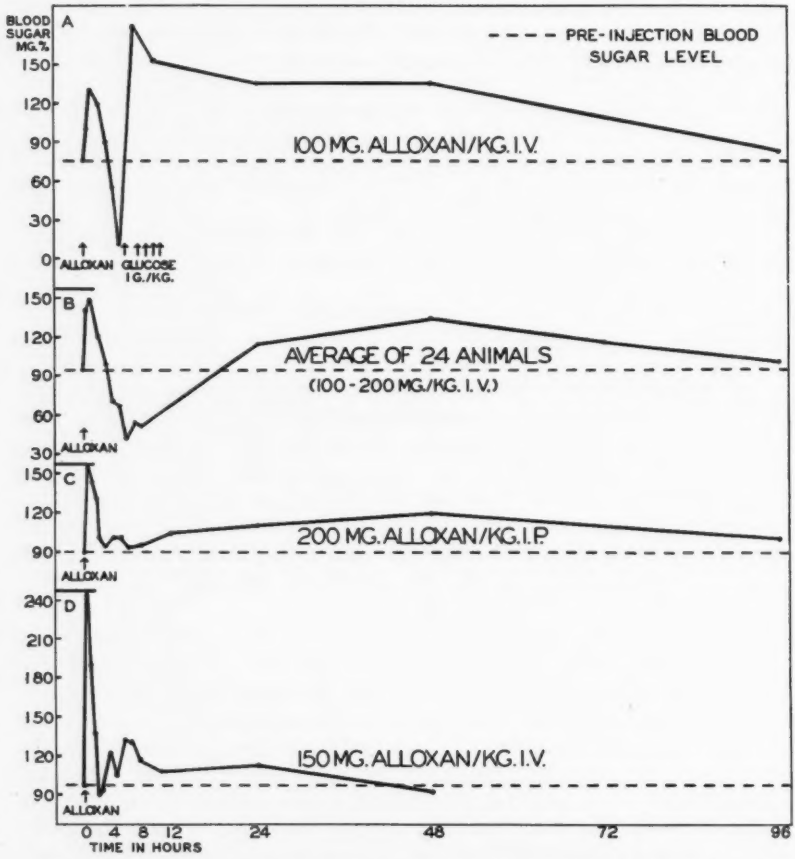


FIG. 1. Blood sugar curves of guinea pigs following alloxan administration. (A) Animal given 100 mg. alloxan per kilo intravenously; fasted prior to injection; received glucose at intervals between 5 and 11 hours to offset the extreme hypoglycemia. (B) Averages of blood sugar values obtained from 24 animals at intervals after intravenous alloxan administration. (Values for some animals which later died, either in hypoglycemia or within the first 48 hours, as well as many which were sacrificed at various times, are included in the averages.) (C) Animal fasted and given 200 mg. alloxan intraperitoneally; note absence of hypoglycemic phase. (D) Animal resistant to the action of alloxan (150 mg. per kilo intravenously, fasted); note absence of hypoglycemic and second hyperglycemic phases. Dotted lines indicate pre-injection blood sugar levels.

TABLE 1. INFLUENCE OF PRE-INJECTION FASTING (14 TO 18 HOURS) ON THE EFFECT OF VARIOUS DOSAGES OF ALLOXAN IN THE GUINEA PIG

Am't of alloxan per kg. body wt.	Mode of administration	No. of animals	Fasted or non-fasted	Response	
				Positive (No. of animals)	Negative (No. of animals)
(mg.)					
100	Intravenous	5	Fasted	4	1
		5	Non-fasted	1	4
150	Intravenous	17	Fasted	16	1
		5	Non-fasted	1	4
200	Intraperitoneal	3	Fasted	3	0
		1	Non-fasted	0	1
200	Intravenous	26	Fasted	24	2
		5	Non-fasted	5	0

with a high of 125 mg. and a low of 60 mg. The average blood sugar level for 16 normal non-fasted animals was 96 mg. per cent with a range of from 82 to 107 mg.

The blood sugar response of guinea pigs during the first 24 hours following an adequate dose of alloxan is similar to that of other animals (Figure 1 B). The peak of the initial hyperglycemia is reached at about 1 hour but is somewhat less marked than in most species. The mean blood sugar level at 1 hour in this series was 148 mg. per cent.

The severity and duration of the hypoglycemic phase varied in different animals. The blood sugar level had fallen below normal by 4 hours after the alloxan injection in most cases. Some recovered spontaneously from an extremely low blood sugar (as low as 16 mg.

TABLE 2. BLOOD SUGAR VALUES OF GUINEA PIGS DURING THE SECOND HYPERGLYCEMIC PHASE (1–4 DAYS) FOLLOWING AN EFFECTIVE DOSE OF ALLOXAN AND DURING THE SAME PERIOD FOLLOWING AN INEFFECTIVE DOSE

Dosage of alloxan	Route of adm.	Fasted or non-fasted (14–18 hrs.)	No. of animals	Days after alloxan	Blood sugar level mg./100 cc. blood		P*
					Average $\pm\sigma$	Dif. from normal	
(mg.)							
None		Fasted	63		95 ± 14		
100 or 200	IV	Fasted	6	1	136 ± 10	+41	<0.01
200	IP	Fasted	4	1	138 ± 12	+43	<0.01
100 or 200	IV	Fasted	6	2	139 ± 19	+44	<0.01
200	IP	Fasted	3	2	137 ± 1	+42	<0.01
200	IV	Fasted	2	3	127 ± 17	+33	<0.01
200	IP	Fasted	3	3	124 ± 2	+29	<0.01
100	IV	Fasted	1	4	83	−12	0.4
200	IP	Fasted	1	4	121	+26	0.07
100 or 150	IV	Non-fasted	11	1	113 ± 18	+18	<0.01
100 or 150	IV	Non-fasted	5	2	113 ± 9	+18	<0.01
100	IV	Non-fasted	3	3	108 ± 11	+13	0.11
100	IV	Non-fasted	1	4	88	− 7	0.6

* Based on the standard error of the mean of that group. A P value of less than 0.01 indicates a significant elevation of the mean of the blood sugar values of the group as compared with the normal fasting level.

%), whereas others required glucose administration to counteract the hypoglycemic convulsions. A mild but definite hypoglycemia was still present in a few animals at 24 hours.

In most cases, the blood sugar level was slightly but significantly elevated by 24 hours (Table 2). The upward trend continued very gradually until the second, or occasionally, the third day. The maximum in most animals was reached by 48 hours, and in no instance did this maximum exceed 174 mg. per cent.

Table 2 shows the mean blood sugar values of guinea pigs on the 4 successive days following alloxan injection. Instead of remaining elevated as in other species, the blood sugar returns to its normal level by the fourth or fifth day. As far as could be determined, the animals then maintained a normal fasting blood sugar level indefinitely (up to 6 months in one case). This, together with a perfectly healthy appearance and a normal weight gain, indicated that these animals had completely recovered from the pancreatic effects of alloxan.

There are some guinea pigs which are resistant or refractory to the action of alloxan. These animals (Figure 1 D) show only the early hyperglycemic fluctuation. As might be expected from the absence of both the hypo- and the second hyperglycemic phases, these animals fail to show any pancreatic lesions.

Several animals receiving less than the 300 mg. dosage died between 24 and 72 hours following injection of alloxan. The cause of death is not definitely known. That the blood sugar level was not responsible in all cases is quite certain. In two instances when blood samples were taken at 24 hours, shortly before the death of the animals, there was still a mild hypoglycemia; in two others the blood sugar level was normal at 48 hours, and a third group of 2 showed a mild hyperglycemia at that time. Those animals from which the pancreas was preserved and studied showed islet lesions commensurate with the dosage of alloxan. Some died during the night, however, and postmortem changes had progressed so far that accurate histological observations could not be made. It has been noted that alloxan is more toxic to the liver and kidney in the guinea pig than in most other animals,[3] and it seems probable that the death of these animals may be referable to injury of either or both of these organs.

Histology of the pancreas following alloxan administration

Observations made on the histology of the normal guinea pig pancreas are in accord with those of other investigators (Bensley and Woerner, 1938; Gomori, 1939). The lesions produced in the pancreatic islets by an effective dose of alloxan are similar to those found in other species.

Group 1. This group of 5 guinea pigs was fasted overnight prior to being given 100 mg. alloxan per kilo intravenously. The animals were

[3] Observations to be published elsewhere.

sacrificed or tissues taken at biopsy at 2, 5½, 7 and 10 hours and 6 months. Histological examination of the tissues shows a minimal effect from the alloxan injections.

At 2 hours a few degenerating B cells are found in most of the larger islets. They present a typical picture of early necrosis: darkly-staining, shrunken cells with pyknotic nuclei (Figure 6). These occasional degenerating cells are scattered in the islets and can be differentiated clearly from the normal B cells. Neither the degree of granularity nor the position in the islet seems to have been a factor in determining which cells were affected. A few dividing B cells were found (not more than 1 in an islet) as well as several mitotic figures in the small ducts and acini.

By 7 hours the lesions are somewhat more extensive. More of the B cells, but still less than half, show degenerative changes. Pyknotic nuclei and fragments of chromatin material are present in many cells. A few cell membranes appear to have ruptured. The spaces left by shrunken B cells, together with the capillaries, gives the islets a honeycomb appearance (Figure 7). The remainder of the B cells and the A cells are unaffected.

The animal which was sacrificed at 10 hours showed no lesions in the islets nor was there any hypoglycemia up to that time.

The pancreas of an animal taken at biopsy 6 months after alloxan injection showed complete recovery and possibly an hypertrophy of the islet tissue. Both the size of the B cells and the proportion of B to A cells appear to be increased relative to the normal. These factors combined give the impression of an excess of insulin-producing tissue. This animal's blood sugar curve during the 4 days following alloxan administration may be seen in Figure 1 A.

Group 2. Four guinea pigs given 100 mg. alloxan per kilo intravenously without previous fasting showed no islet cell lesions 10 minutes, 1 day, 3 days and 6 days after the injection.

Group 3. Twenty-six guinea pigs fasted overnight and subsequently given intravenous injections of 150, 175 or 200 mg. alloxan per kilo were sacrificed or tissue taken at biopsy at intervals from 2½ hours to 16 days. For purposes of description a few representative stages during the degeneration and repair of the islet tissue have been selected.

At 2½ hours extensive necrosis involves most of the B cells. The A cells appear normal.

Necrosis is slightly more advanced at 3½ hours, with coalescence of some of the B cells (Figure 8). The B granules stain less brightly—a dull gray-blue instead of the normal clear blue with the chrome hematoxylin-phloxin stain. There is some slight fragmentation of the cytoplasm of the A cells.

By 6 hours, in addition to the many degenerating B cells as described above, there are some in which the pyknotic nuclei have begun

to fragment and liquefy. The appearance is somewhat similar to that of a cell undergoing mitosis. However, the two can be differentiated with certainty. The B cell in metaphase has a large, central, densely chromatic figure with irregular outlines; the cell body is large and lightly granulated. The degenerating B cell contains several small, individual, densely chromatic fragments; the cell body is shrunken and small with deeply staining, closely packed granules (cf. Figures 5 and 9).

Degenerative nuclear changes are more advanced at 12 hours. A few of the nuclei are pyknotic; many show liquefaction and fragmentation (Figure 10); and some have already been partially removed, leaving only a few very small chromatin fragments in the center of a shrunken cell.

At 24 and 28 hours, there is evidence of dissolution and removal of many of the necrotic B cells: large areas in the islets appear as a blue-gray background with an occasional pyknotic nucleus and a few scattered nuclear fragments (Figure 11). The degeneration of the B cells is more advanced in some islets than in others. Even the B cells in the smallest islets and an occasional B cell situated among the acinar cells in the parenchyma have been affected. Nevertheless, there still remain a few normal B cells which are scattered in small groups among the degenerating ones. There are distinct spaces in the islets—enlarged pericapillary spaces—usually with a few granules scattered in them. The A cells in general appear normal, although the cytoplasm of a few is somewhat fragmented or vacuolated.

During the next 24 hours nearly all of the necrotic B cells disappear; the remainder appear as small areas of nearly homogeneous blue-gray staining material with an occasional pyknotic nucleus (Figure 12). These areas are found in some of the larger islets, especially in regions near the capillaries. The islets are otherwise filled with A and B cells. The derivation and appearance of these B cells have been studied and will be reported in detail. The number of very small islets appears to be increased. These islets are made up almost exclusively of B cells. As a consequence, the proportion of B to A cells appears to be greater than in the normal.

There is no essential change in the appearance of the islet tissue between the second and third days except for the almost total absence of any cytoplasmic or nuclear remains of necrotic cells in the majority of islets on the third day. There are still, however, a few pyknotic nuclei in the largest islets.

By 4 days the islet tissue is normal in appearance, with no cellular debris and no evidence of shrinkage in the islets (Figure 13). Both the B and A cells are large and heavily granulated. There are many small islets, composed mainly of B cells. In all of these animals the typical triphasic blood sugar curve was obtained during the first two days.

TABLE 3. RESPONSE OF GUINEA PIGS TO INTRAVENOUS ALLOXAN INJECTION: CORRELATION OF HISTOLOGICAL AND BLOOD SUGAR CHANGES

Animal	Dosage of alloxan per kg. body wt.	Time between successive alloxan injections	Fasted or non-fasted (14 to 18 hours)	Time between alloxan adm. and autopsy or biopsy	Presence of degenerating B cells[1]	Initial hyperglycemia[2]	Hypoglycemia[3]	Transient hyperglycemia[4]
	(mg.)							
No. 20	100		Fasted	2 hours	++	+++		
No. 18	100		Fasted	5½ hours	+	+		
No. 19	100		Fasted	7 hours	++	++	+++	
No. 15	100		Fasted	10 hours	−	++	−	
No. 17	100		Fasted	6 months	−	++	++++	+
No. 32	100		Non-fasted					−
	100	1 day	Non-fasted					−
	100	1 day	Non-fasted					±
	100	1 day	Non-fasted	10 minutes	−			
No. 3	100		Non-fasted				−	+
	100	2 days	Non-fasted	24 hours	−		−	+
No. 21	100		Non-fasted					−
	100	2 days	Non-fasted	3 days	−			−
No. 24	100		Non-fasted				−	−
	100	2 days	Non-fasted	6 days	−			−
No. 40	200		Fasted	2½ hours	++++		++++	
No. 44B$_2$	200		Fasted	2½ hours	+++		+	
No. 48	200		Fasted	2½ hours	+++		++++	
No. 13	150		Fasted	3½ hours	++++	++	++++	
No. 50	200		Fasted	5 hours	++++			
No. 55	175		Fasted	11 hours	++++			
No. 7	200		Fasted	24 hours	++++	++	++++	
No. 11	150		Fasted	24 hours	++++	++	+	
No. 59	200		Fasted	28 hours	++++	++	+++	
No. 43	200		Non-fasted	4 days	−		++++	−
No. 39B	200		Fasted	4 days	−		++++	+
No. 44B$_1$	200		Fasted	4 days	−		+	+
No. 53	200		Fasted	4 days	−		++++	+
No. 51	200		Fasted	5 days	−			±
No. 39	200		Fasted	12 days	−		++++	+
No. 80	300		Fasted	2 hours	+++			
No. 63	300		Fasted	4 hours	++		++++	
No. 71	300		Fasted	9 hours	++		++++	
No. 67	300		Fasted	24 hours	++		++++	
No. 68	300		Fasted	50 hours	+++		++++	

[1] Designations indicating number of degenerating B cells:
− none of the B cells degenerating
+ few of the B cells degenerating
++ less than ½ of the B cells degenerating
+++ more than ½ of the B cells degenerating
++++ practically all of the B cells degenerating (maximal response for guinea pig)

[2] Designations used for indicating degree of initial hyperglycemia:
+ blood sugar 25–40 mg. % above fasting level
++ blood sugar 41–60 mg. % above fasting level
+++ blood sugar 61–80 mg. % above fasting level

[3] Designations used for indicating degree of hypoglycemia:
− no lowering of blood sugar within first 10 hours
+ blood sugar 0–25 mg. % below fasting level
++ blood sugar 26–40 mg. % below fasting level
+++ blood sugar 41–60 mg. % below fasting level
++++ blood sugar 61–90 mg. % below fasting level

[4] Transient hyperglycemia:
Definite hyperglycemia = more than $2\frac{1}{2}\sigma$ above normal blood sugar level (95 ± 14 mg.%) = >130 mg.%
Questionable hyperglycemia = $2-2\frac{1}{2}\sigma$ above normal blood sugar level = 123–130 mg.%
No hyperglycemia = < 2σ above normal blood sugar level = <123 mg.%

Recovery of islet tissue following alloxan administration

In view of the replacement and even an apparent increase in the number of B cells in those animals which recovered from the alloxan, it seemed of interest to determine the ratio of B to A cells before and after alloxan treatment. (Counts of the total number of pancreatic islets were not made in any of the animals, since it was impossible in those in which tissue was taken at biopsy.) The percentage of B cells was determined in 6 untreated control and 9 experimental animals which had been sacrificed or tissues taken at biopsy at 4 days to 6 months after alloxan injection.

In the untreated guinea pig the number of B cells (based on counts

of 3000 or more cells and expressed as the percentage of the total number of islets cells) varies within the small range of 77.8% to 79.3% from one animal to another. After recovery from alloxan injection, the percentage of B cells ranges from 79.5% to 89.3% of the total. Statistical analysis (Fisher, 1946) shows this to be a significant increase over the normal in all but one case.[4] The difference between the means of the control and experimental groups is more than 27 times the standard error and is, therefore, significantly outside the range of possible chance variation.

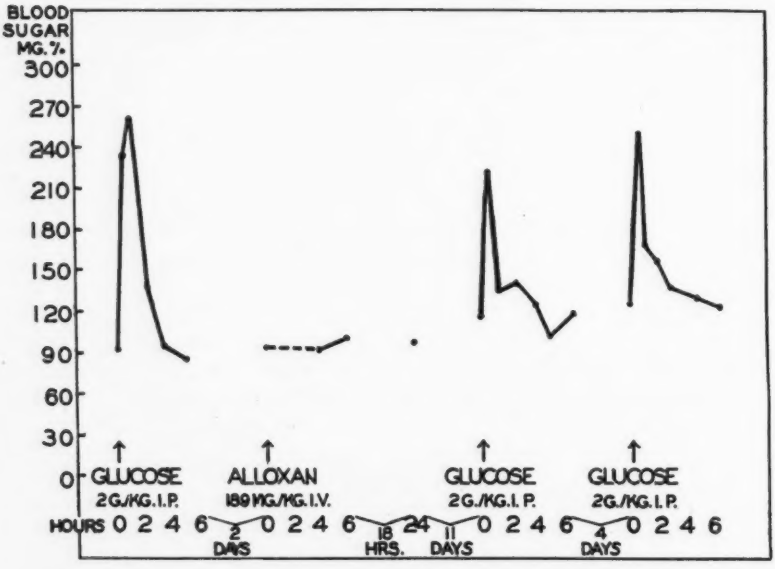


FIG. 2. Blood sugar curves of a guinea pig (#39), representative of those animals which are only mildly affected by alloxan. A normal glucose tolerance test, followed by an ineffective dose of alloxan and two subsequent glucose tolerance tests (12 and 16 days after alloxan).

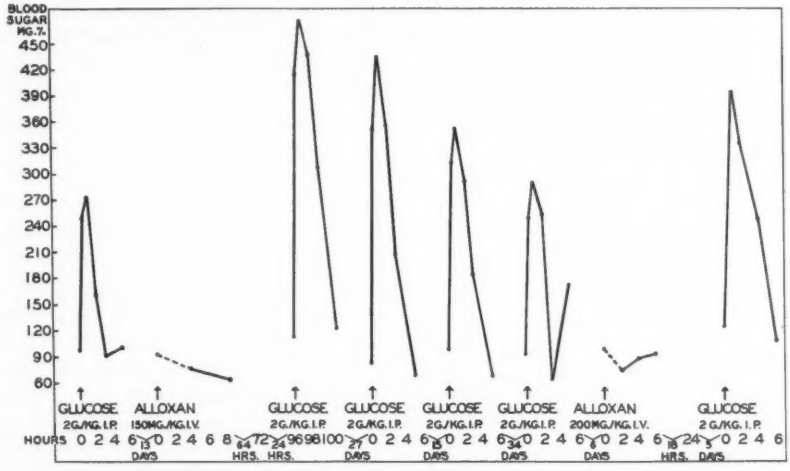
Glucose tolerance after alloxan

The glucose tolerance curve for the normal guinea pig shows considerable variation, even in the same animal from week to week. From the 29 curves obtained from untreated animals, that type which occurred most frequently has been selected as the basal or normal curve. It corresponds very closely with the curve resulting from averaging the values for the 29 individual curves. It also corresponds closely with the normal glucose tolerance curves for rats (Shipley and Rannefeld, 1945). Two such "normal" curves may be seen in Figures 2

[4] In this animal the blood sugar curve showed a very slight hypoglycemia, and there was a normal tolerance for glucose on the third day.

and 3, the pre-alloxan curves. The peak of the curve occurs at 1 hour (occasionally at ½ hour) after glucose administration; and by 3 hours, the blood sugar level is either normal or nearly normal. Any pronounced elevation or prolongation of the curve is considered as indicating an abnormal state of carbohydrate metabolism.

In this experiment, the following procedures were carried out: (1) at least one normal glucose tolerance test was performed on each animal; (2) two or more days later, alloxan was administered intravenously (150 to 200 mg. per kilo); and (3) on the third or fourth day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GLUCOSE 2G./KG. I.P.
ALLOXAN 150MG./KG. I.V.
GLUCOSE 2G./KG. I.P.
GLUCOSE 2G./KG. I.P.
GLUCOSE 2G./KG. I.P.
GLUCOSE 2G./KG. I.P.
ALLOXAN 200MG./KG. I.V.
GLUCOSE 2 G./KG. I.P.
HOURS 0 2 4 6 13 DAYS 0 2 4 6 8 44 72 HRS. 24 96 98 100 HRS. 27 DAYS 0 2 4 6 35 DAYS 0 2 4 6 34 DAYS 0 2 4 6 6 DAYS 0 2 4 6 18 24 HRS. 5 DAYS 0 2 4 6

FIG. 3. Blood sugar curves of a guinea pig (#60), representative of those animals in which there is extensive islet damage following alloxan administration. A normal glucose tolerance test, followed by an effective dose of alloxan and four successive glucose tolerance tests (4, 32, 48 and 83 days later), a second alloxan injection (ineffective, as shown by biopsy at 28 hours) and glucose tolerance test on sixth day.

following alloxan, and at various intervals thereafter, glucose tolerance tests were performed to determine the extent of the animals' recovery. Of the 8 animals thus studied, 6 showed a decreased tolerance for glucose within 4 days after alloxan, 1, in which there had been only very slight blood sugar fluctuations after alloxan, showed a normal glucose tolerance curve, and in 1 animal there was a questionable response.

Figures 2 and 3 show the alloxan and glucose tolerance curves of two animals selected because they are representative of two groups: that in which the animals are only mildly affected by alloxan (Figure 2) and that in which the islet tissue of the pancreas is more severely damaged (Figure 3).

Following an ineffective dose of alloxan, the animal represented in Figure 2 showed no hypoglycemia, which indicated little if any islet damage. The glucose tolerance of this animal, however, was increased

12 and 16 days later, as evidenced by the low level of the blood sugar at 1 hour. Whatever slight necrosis there may have been in the B cells evidently served as a stimulus to hypertrophy of the insulin-producing tissue.

In the animal represented in Figure 3, alloxan administration resulted in the usual hypoglycemia at 4 to 8 hours, and a mild hyperglycemia remaining on the third day. The fasting blood sugar had returned to nearly the normal level by the fourth day when the first of a series of 4 glucose tolerance tests was done. This curve shows an extremely low carbohydrate tolerance. The 3 successive glucose tolerance tests (performed 27, 43 and 78 days later) exhibit a gradual return to normal. It is significant to note that at no time during this period was the fasting blood sugar level elevated. A biopsy of the pancreas 8 days following the last glucose tolerance test and 28 hours after a second injection of alloxan showed healthy and abundant islet tissue with heavily granulated B cells and an increase in the proportion of B to A cells.

The blood sugar curve resulting from the second injection of alloxan (7 days after the last glucose tolerance test) shows neither the pronounced hypoglycemia nor the hyperglycemia indicative of extensive B cell necrosis. Biopsy of the pancreas at 28 hours confirmed the absence of islet damage. And yet on the sixth day following this alloxan injection there was once again a very low tolerance for injected glucose (Figure 3). Sections of the liver taken at autopsy show lesions which indicate that this organ may have been responsible for the decreased tolerance for glucose.

DISCUSSION

Reports on the effects of administration of alloxan to the guinea pig are few in number and somewhat conflicting. Goldner and Gomori (1944 a) were unable to establish a diabetogenic dose of alloxan in this species, since degenerative lesions in the pancreas were found only in those animals which died within 24 hours after injection. Woerner (1947) found that intravenous injections of 50 to 200 mg. per kilo produced more extensive cell lesions in the large islets than in the small ones. His animals were all sacrificed at 24 hours, however, and no blood sugar changes were reported. Saviano and De Franciscis (1947) found that after intravenous injection of 50 to 450 mg. dosages there was a prolonged hypoglycemia which in the majority of cases was fatal. In two of the animals which recovered from the hypoglycemia there was glycosuria accompanying blood sugar levels of 123 to 147 mg.% at 14 and 18 days. The blood sugar of these two animals was 100 and 117 mg. with no glycosuria at 21 and 24 days. Histologically, these investigators found varying degrees of necrosis in the pancreatic islets. Normal and degenerating cells were found in the same islets. Griffiths (1948) was unable to produce hypoglycemia in normal guinea pigs by subcutaneous or intracardial injections of 200 mg.

alloxan. The same dosage, however, did result in hypoglycemia within 4 hours if injected directly into the pancreas. Necrosis of both islet and acinar tissue was noted in the region of the injection. Also, Griffiths noted that a similar hypoglycemia could be produced after intraperitoneal injection in guinea pigs whose blood reduced-glutathione level had been lowered by a methionine and cystine deficient diet. There was no mention of either a temporary or permanent hyperglycemia in any of the experiments. The fasting blood sugar levels of his animals were in the range of 115 to 135 mg.% (estimated from curves in which averages were reported).

West and Highet (1948) observed what they considered to be a normal blood sugar level in all of their animals 48 hours after injection of from 150 to 1000 mg. of alloxan, the higher dosages being given subcutaneously or intraperitoneally. Some of the post-alloxan blood sugar levels (130 to 170 mg.%) reported by these authors are above the range of the normal values for the animals used in the present study and would, therefore, be considered definitely hyperglycemic. Charalampous and Hegsted (1949) observed no significant changes in either the blood sugar or the islet tissue following 200 mg. dosages given intravenously. Blood sugar levels as high as 150 mg.% were considered within "the normal range for the guinea pig." No histological studies were made earlier than two days. Dosages of 225 mg. or more of alloxan were found to be fatal within three days and to produce islet lesions varying from cloudy swelling to necrosis and disappearance of the B cells.

Lazarow (1946) suggested the importance of the blood reduced-glutathione level as a factor in the susceptibility of various species and individuals to the action of alloxan. Griffiths (1948) has shown that the guinea pig normally has a higher blood glutathione level than the rabbit, and that in both the guinea pig and the rabbit susceptibility to the hypoglycemic action of alloxan depends upon a low concentration of reduced-glutathione in the blood. It is not impossible that some of the discrepancies in the reports in the literature on the effectiveness of alloxan in guinea pigs may be explained by a difference in the amount of glutathione available from the different diets used by various investigators. Blood reduced-glutathione levels have not been determined in any of the animals used in the present study. The animals have been kept on a diet of Rockland Guinea Pig Diet supplemented with fresh lettuce and carrots.

The enhancing influence of fasting on the action of alloxan has been observed in several species: rabbits (Jacobs, 1937; Hughes, Ware and Young, 1944), rats (Kass and Waisbren, 1945) and dogs (Manhoff and De Loach, 1948). From the results presented here it is apparent that the guinea pig is more sensitive to alloxan when fasted than when fed but that it is not made permanently diabetic in dosages as high as 300 mg. per kilo even when fasted. Those animals in which pancreatic lesions are produced either recover completely or they die in

the first 24 to 72 hours after injection. The maximal response to alloxan injection was found to be necrosis of practically all of the B cells in the pancreatic islets with the accompanying triphasic blood sugar fluctuations up to approximately the fourth day. That amount of alloxan which produces extensive islet cell lesions or these blood sugar changes in at least 80% of the animals has been designated as an "effective dose".[5] This dose is 200 mg. alloxan per kilo given intravenously to either fasted or non-fasted guinea pigs. Smaller doses (100 and 150 mg.) are also effective providing the animals are fasted prior to injection.

Some investigators reporting the ineffectiveness of alloxan administration in the guinea pig have apparently been misled by the rapid recovery of those animals not killed by the toxic action of the drug on other organs. No observations have previously been made on the histological appearance of the islets during the first 24 hours and no blood sugar analyses except for the few reported by Saviano and De Franciscis (1947) and Charalampous and Hegsted (1949) earlier than 48 hours. Also, the effect of pre-injection fasting in relation to the effectiveness of small dosages of alloxan in the guinea pig has not been reported. The 150 mg. intracardial doses reported as ineffective by West and Highet (1948) in non-fasting guinea pigs were found in the present study to be quite effective intravenously if the animals were fasted 14 to 18 hours prior to injection.

The normal fasting blood sugar level in guinea pigs reported by Banerjee (1943) is 83 to 153 mg.%. Charalampous and Hegsted (1949) have given 85 to 150 mg.% as the normal range. The fasting blood sugar levels in the present experiments ranged from 60 to 125 mg.%, while the non-fasting values ranged from 82 to 107 mg.%. The reason for the greater variation in the fasting animals is unexplained. Analysis of the figures does not indicate that the larger number of samples (63 as compared with 16) is entirely responsible. The mean blood sugar values for the two groups are, however, almost identical: 95 mg.% (fasting) and 96 mg.% (non-fasting). In view of this fact, it was felt that both fasting and non-fasting blood sugar values should be included in the table of results for the 4 days following alloxan injection (Table 2).

The blood sugar changes in the guinea pig following alloxan administration reported here are, in general, similar to those in other animals up to the third or fourth day. The initial hyperglycemia reaches a peak of 150 to 400 mg.% at about 2 hours in rabbits (Dunn, Kirkpatrick, McLetchie and Telfer, 1943; Hard and Carr, 1944; Goldner and Gomori, 1944a), and similarly in rats, 130 to 240 mg.% at 2 hours (Duff and Starr, 1944). In the guinea pigs used in these

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[5] This term is used in lieu of "diabetogenic dose" since the sustained hyperglycemia which serves as the criterion for diabetes (Goldner and Gomori, 1944a; Leech and Bailey, 1945; Levey and Suter, 1946) is not present in the guinea pig.

experiments, the peak of the initial hyperglycemia was reached at about 1 hour, the average value at this time being 148 mg.%, and the maximum blood sugar value recorded during the initial hyperglycemic phase was 245 mg.%.

Convulsions during the hypoglycemic phase are not as pronounced in the guinea pig as in rabbits and monkeys. Nevertheless, the blood sugar often reaches extremely low levels during this phase. Some guinea pigs do not exhibit any definite symptoms when the blood sugar falls as low as 16 mg.%, whereas others require repeated glucose administration to alleviate the weakness, hyperirritability and occasional convulsions, which are the signs of extreme hypoglycemia in the guinea pig.

The mild and transitory nature of the second hyperglycemic phase (the third phase of the triphasic response) was constantly found in the animals used in this study. From an average high of 138 mg.% on the second day, the blood sugar invariably returned to the normal level by the fourth or fifth day, and remained there indefinitely. This is in contrast to most other species where recovery of the pancreatic islands after a dosage of alloxan sufficient to injure the B cells is exceptional. Dunn and McLetchie (1943) and Janes (1947) have reported recovery of mildly diabetic rats, but only after a month or more following alloxan treatment. Recovery has also been observed in the rabbit at 20 days (Brunschwig and Allen, 1944), in a very mildly diabetic dog at 3 days (Carrasco-Formiguera, 1944) and in 50% of mice at the end of 6 weeks (Waisbren, 1948). Two important differences are apparent here. First, in most species only a few animals which show very mild effects of alloxan return to normal, while in the guinea pig those with the most pronounced blood sugar changes recover as completely as those which are only mildly affected. Secondly, recovery when it occurs in other species takes a much longer period of time than in the guinea pig.

The lesion produced in the pancreatic islets of the guinea pig by alloxan injection is, in general, the same as in other species. However, there are certain features in which the guinea pig differs from other animals: (1) a few of the B cells remain uninjured (a result which occurs occasionally but not consistently in other species); (2) there is no exhaustion and hydropic degeneration of those B cells which remain; (3) there is no replacement of B cells by agranular cells; (4) there is no shrinkage and ultimate disappearance (fibrosis) of the islets; (5) there is complete restoration of islet tissue within 4 days after alloxan administration.

From the data and descriptions already presented, it may be seen that there is a definite correlation between the blood sugar changes and the histological appearance of the pancreas. The initial hyperglycemia can be considered an extrapancreatic phenomenon (Hughes, Ware and Young, 1944; Goldner and Gomori, 1944b; Houssay, Orias and Sara, 1945). We have, then, briefly: (1) an hypoglycemia becoming

progressively more severe during the period of B cell degeneration (from 4 to 8 hours or longer) when the insulin is being released from the dying B cells; (2) a mild hyperglycemia during the period of repair when the pancreas is producing less insulin (1 to 3 days); and (3) a return to the normal blood sugar level with the restoration of the islet tissue.

Although recovery of the islet tissue has been reported in other species (Brunschwig and Allen, 1944; Ruben and Yardumian, 1945), it is rare. In the rat, rabbit and monkey the B cells are usually replaced by agranular cells of an unidentifiable type, and in the dog there is a complete disappearance of the cells (Goldner and Gomori, 1944 a). In those rabbits in which spontaneous recovery occurs, there is a proliferation of the few B cells which remain in the islets. However, according to Goldner and Gomori (1944 a), these cells do not regain their normal morphology (heavy granulation) even after months.

It will be recalled that at 24 hours the pancreas of the alloxan-treated guinea pig shows very few normal B cells. By 4 days the islet tissue is abundant. Both B and A cells are large and heavily granulated, the former being more uniformly packed with deeply-staining granules than in the normal. In addition to the large, medium-sized and small islets which are normally found in the guinea pig pancreas, there are a great many small groups of islet cells, almost exclusively B cells, scattered throughout the parenchyma.

The many small islets and the large, heavily granulated B cells present immediately following recovery are also seen 6 months after alloxan treatment.

It is interesting to note that in those animals which remained active and healthy during the first day following alloxan administration, the nuclear and cytoplasmic remains of the necrotic B cells were almost entirely removed within the first 18 hours. The pyknotic or fragmented nuclei disappeared earlier than the cytoplasmic debris in many cases.

It has been pointed out earlier that although there is a com-

PLATE 1. EXPLANATION OF FIGURES

Photomicrographs of sections through the pancreas of guinea pigs. *A*, nucleus of A cell; *B*, nucleus of B cell; c, capillary; *M*, mitotic figure; *PB*, pyknotic nucleus of B cell; *Z*, acinar cell of pancreas. All sections cut at 4 μ and stained with chrome hematoxylin-phloxin.

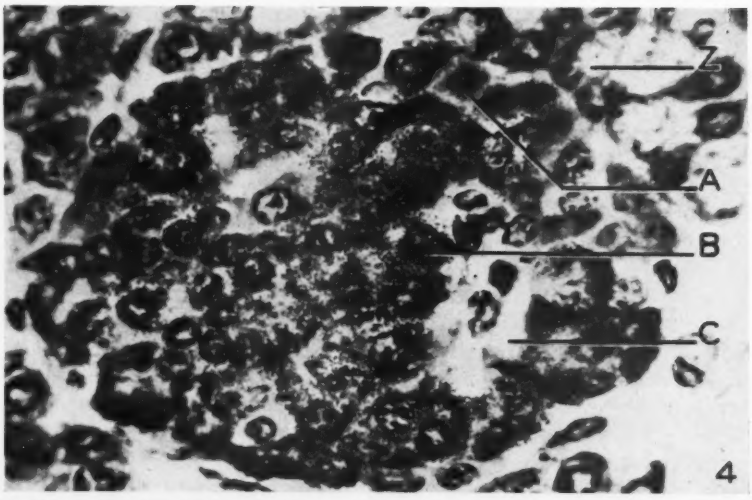
4. A normal, untreated guinea pig showing an island of Langerhans and adjacent acinar tissue. ×933.

5. A normal control guinea pig injected with colchicine (0.3 mg. per kilo) 9 hours before autopsy; a dividing B cell has been arrested in metaphase. ×933.

6. Two hours after intravenous injection of 100 mg. alloxan per kilo; several B cells are in early stages of degeneration. ×933.

7. Seven hours after intravenous injection of 100 mg. alloxan per kilo; B cell necrosis is further advanced than at 2 hours. ×933.

8. Alloxan-treated (150 mg. per kilo intravenously); 3½ hours; a larger percentage of the B cells are degenerating than with the 100 mg. dosage. ×933.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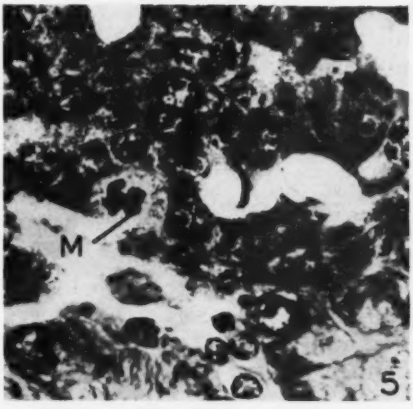
4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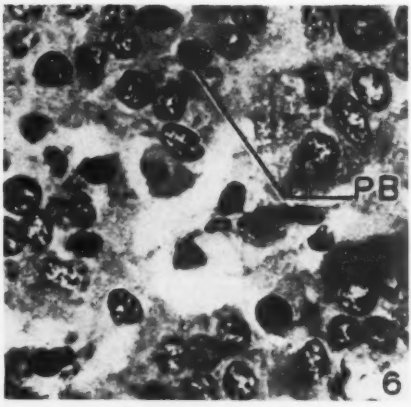
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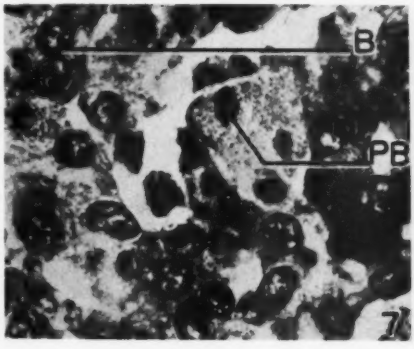
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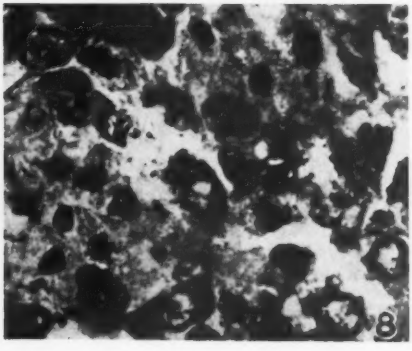
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plete restoration of B cells after alloxan injection, nevertheless, the guinea pigs later had a decreased glucose tolerance. The glucose tolerance test has been used as a measure of latent diabetes following small doses of alloxan (in rats by Shipley and Rannefeld, 1945; in rabbits by Martinetti and Andreani, 1946) and of the degree of recovery after a diabetogenic dose of alloxan (Goldner and Gomori, 1944 a). Despite a normal fasting blood sugar under these circumstances, the glucose tolerance curve is typically diabetic. The two outstanding features of these curves are: (1) the high degree of elevation and (2) the extreme prolongation as evidenced by the delay beyond 5 hours in the return to the normal level.

Soskin (1944) has compared the glucose tolerance curves of mildly diabetic individuals and those with mild liver disease. He finds the two curves similar in all but one respect: the diabetic curve remains elevated above the pre-injection level much longer than that of liver disease. The difference is pronounced enough with his technique to be considered diagnostic. The glucose tolerance curve of the guinea pig following alloxan administration much more closely resembles that resulting from liver damage than that of diabetes. The 5-hour sample is invariably in the range of the pre-injection level. This is in contrast to the curves for rats and rabbits after repeated small doses of alloxan which are typically diabetic, with the 5-hour sample decidedly elevated (in the region of 200 mg.%).

Liver damage following alloxan administration has been reported by many investigators (Goldner and Gomori, 1943; Duff and Starr, 1944; Bailey, Bailey and Leech, 1944; Lackey, Bunde, Gill and Harris, 1944; Biasotti and Porto, 1945; Ruben and Yardumian, 1945; Lazarow and Palay, 1946; Herbut, Watson and Perkins, 1946), and it has been suggested that alloxan may have a direct effect on the liver as well as on the pancreas (Goldner and Gomori, 1943; Houssay, Orias and Sara, 1945; Herbut, Watson and Perkins, 1946).

In view of the decreased glucose tolerance in spite of the rapid and

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PLATE 2. EXPLANATION OF FIGURES

Photomicrographs of sections through the pancreas of guinea pigs. *A*, nucleus of A cell; *B*, nucleus of B cell; *C*, capillary; *N*, nuclear fragments of necrotic B cell; *O*, cytoplasmic debris of necrotic B cell; *PB*, pyknotic nucleus of B cell; *Z*, zymogen granules or acinar cell of pancreas. All sections cut at 4 μ and stained with chrome hematoxylin-phloxin.

9. Alloxan-treated (200 mg. per kilo intravenously); 6 hours. ×933.

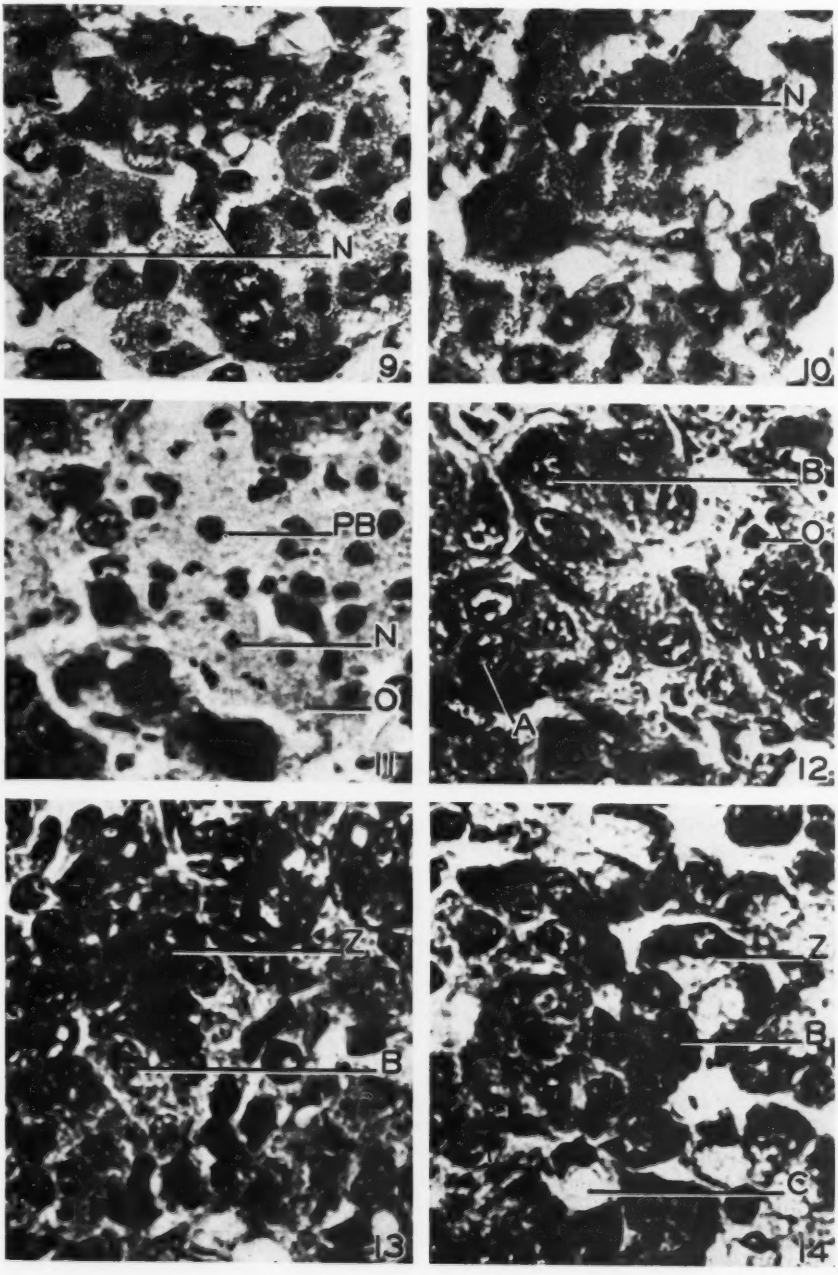
10. Alloxan-treated (200 mg. per kilo intravenously); 12 hours. ×1008.

11. Alloxan-treated (150 mg. per kilo intravenously); 24 hours. ×1008.

12. Two days (48 hours) after alloxan (150 mg. per kilo intravenously); islet contains bits of cytoplasmic debris which are the remains of the necrotic B cells scattered among the normal A and B cells. ×1008.

13. Four days after alloxan (200 mg. per kilo intravenously); no evidence of degenerating B cells remains; B cells are large and heavily granulated. ×1008.

14. Following recovery from the effects of alloxan administration (3 months after 150 mg. per kilo intravenously); islet contains large, heavily granulated A and B cells. ×1008.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complete recovery of the islet tissue in the guinea pig, the similarity of the glucose tolerance curve to that resulting from mild liver disease, the fact that the glucose tolerance does eventually return to normal, and the presence of liver necrosis in several of the animals from which this organ was preserved, it is suggested that in the guinea pig the liver may be responsible for the prolonged changes in carbohydrate metabolism after alloxan administration. The liver damage is probably reversible in many cases, but may explain the death of some animals during the first 3 days. Liver sections from one animal, #60, taken 3 months after an effective dose of alloxan, show a considerable area of replacement of hepatic cells by scar tissue at the periphery of the lobules. Further studies of the role of the liver in the response of the guinea pig to alloxan are in progress and until completed no definite statement can be made.

The possibility of kidney damage as a factor in the response of some animals to alloxan should not be overlooked. Degeneration of the kidney tubules (either proximal or distal or both) has been reported, particularly as the result of large doses of alloxan (Dunn, Sheehan and McLetchie, 1943; Bailey, Bailey and Leech, 1944; Brunschwig and Allen, 1944; Duff and Starr, 1944; Ruben and Yardumian, 1945). Kidney sections were taken from several of the guinea pigs used in these experiments. All show more or less extensive lesions, particularly in the proximal convoluted tubules. A mild degree of ascites was also noted in a few of the animals.

SUMMARY

Intravenous injection of alloxan produces necrosis of the B cells in the islands of Langerhans of the guinea pig.

The blood sugar changes following intravenous injection are a transient initial hyperglycemia, a more or less severe hypoglycemia, and a second temporary hyperglycemia. The blood sugar level returns to normal by the fourth or fifth day.

An effective dosage is defined as that amount of alloxan (200 mg. per kilo intravenously) which will produce these changes in at least 80% of the animals.

Overnight fasting increases the sensitivity of the guinea pig to the action of small dosages of alloxan.

Complete recovery of the islet tissue takes place within four days following alloxan administration and persists for the period which animals have been kept (6 months).

There is an hypertrophy of insulin-producing tissue, due to the formation of new small islets composed mainly of B cells.

Glucose tolerance is impaired following an adequate dosage of alloxan. The impairment persists with decreasing severity for several weeks. The glucose tolerance curve, however, during this time is dissimilar to a diabetic curve and resembles that resulting from mild liver damage.

Following an ineffective dose of alloxan there may be hypertrophy of the islet tissue without concurrent liver damage, and consequently an increased tolerance for glucose.

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THE EFFECTS OF PTEROYLGLUTAMIC ACID DEFICIENCY AND PTEROYLGLUTAMIC ACID REPLACEMENT ON THE ENDOCRINE GLANDS OF THE IMMATURE CHICK[1]

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INTRODUCTION

THE INTER-RELATIONSHIPS between vitamins and endocrine physiology have been recognized for some time. Unfortunately the role of vitamins in body economy has been considered principally from the standpoint of body growth, and only recently have other criteria been studied. Endocrine substances and vitamins often affect the same general physiological processes either in the same direction or antagonistically. It is necessary, therefore, to differentiate where possible the specific effects of each in any study of either group. One may cite the well known importance of the pituitary gland to somatic growth (Marx and Evans, 1944), the anabolic effects of testoid sterols on protein metabolism (Kochakian, 1946), the effects of adrenal corticoids on carbohydrate and electrolyte metabolism (Ingle, 1944; and Samuels, 1948), and the part played by the thyroid in general growth processes (Drill, 1943).

The report of deleterious effects of pteroylglutamic acid (PGA) deficiency on body growth leads one to question the mechanism involved. Hertz (1945, 1946, 1948) has recently shown that the oviducts of "folic acid" deficient chicks are less able to respond to the stimulating effects of estrogen than those of normal or PGA-treated chicks, and that this effect was not demonstrated in several other B vitamin deficiencies. It became of interest, therefore, to study certain endocrine aspects of PGA-deficient chicks receiving graded levels of PGA.

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[2] Now at the M. D. Anderson Hospital for Cancer Research, University of Texas, Houston.

MATERIALS AND METHODS

White Leghorn chicks which had been "sexed" in order to select only males, were obtained from a commercial hatchery and were placed on the experimental diets at approximately 2 days of age. They were housed in metal battery brooders with wire mesh floors, and were given the experimental diets and water *ad libitum*. The diet was similar to that recently recommended for use in chick assays for the vitamin M group by Day and Totter (1947). Its composition is given below:

Casein, vitamin-free (Labco)	25.0	gm.
Gelatin	10.0	gm.
Corn-starch	52.4	gm.
Cellulose	0.3	gm.
Hydrogenated vegetable oil	0.3	gm.
Cod liver oil	1.3	gm.
Salts (Jones and Foster, 1942)	0.5	gm.
l-Cystine	0.3	gm.
$MnSO_4 \cdot 4H_2O$	0.1	gm.
Choline chloride	0.2	gm.
Thiamine chloride	0.4	mg.
Riboflavin	0.8	mg.
Pyridoxine hydrochloride	0.6	mg.
2-Methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine lactone[3]	0.05	mg.
Calcium pantothenate	1.1	mg.
Nicotinic acid	2.0	mg.
i-Inositol	5.0	mg.
p-Aminobenzoic acid	15.0	mg.
2-Methylnaphthoquinone	0.005	mg.
α-Tocopherol	24.0	mg.
Biotin[3]	0.01	mg.

All chicks were weighed weekly for 4 weeks. Groups I and II received only the basal diet with no PGA added, and were considered to be receiving a PGA-free ration. Groups III to IX, inclusive, received the basal diet with 5, 10, 20, 40, 80, 200, and 1000 μg. of PGA added to each 100 gm., respectively. Group X received Purina "Startena" containing 175 μg. of PGA per 100 gm. as shown by *Streptococcus faecalis* assay.

Hemoglobin, hematocrit, and total and differential blood cell counts were made after 4 weeks on the experiment. The chicks were killed by decapitation as near to the base of the skull as possible in order to preserve intact the entire thymic mass. The hypophyses, thyroids, adrenals, testes, ovaries, and oviducts were removed and weighed to the nearest 0.1 mg. on an analytical balance. The comb and thymic masses were removed and weighed to the nearest mg. on a torsion balance. All tissues but the pituitary glands were preserved in Bouin's solution, embedded in paraffin, sectioned, and stained in hematoxylin and eosin. The pituitary glands were smeared between glass slides, and the smears dried and stored according to the method of Kupperman, Elder and Meyer (1941). Additional details of procedure will be considered in the individual parts of the section to follow.

RESULTS AND DISCUSSION

Gross Organ Weights.—The gross body and organ weights are summarized in Table 1. These figures are the arithmetic means plus or minus the standard errors corrected for small numbers. The number of individual weights from which the various means were calculated

[3] Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

TABLE 1. MEAN GROSS ORGAN WEIGHTS
(±Standard Errors)

Organ	I & II 0 μg. PGA	III 5 μg. PGA	IV 10 μg. PGA	V 20 μg. PGA	VI 40 μg. PGA	VII 80 μg. PGA	VIII 200 μg. PGA	IX 1000 μg. PGA	X Startena (175 μg.)	I	II
Body Weight	117 gm. ±15	122 gm. ±12	163 gm. ±24	201 gm. ±18	267 gm. ±13	291 gm. ±15	319 gm. ±15	281 gm. ±30	339 gm. ±28	118 gm. ±31	117 gm. ±13
Hypophysis Weight	3.2 mg. ±0.2	4.8 mg. ±0.2	4.1 mg. ±0.3	4.9 mg. ±0.5	5.4 mg. ±0.3	5.6 mg. ±0.4	6.1 mg. ±0.4	6.2 mg. ±0.9	6.1 mg. ±0.4	2.7 mg. ±0.1	3.5 mg. ±0.3
Thyroid Weight	7.8 mg. ±1.5	8.3 mg. ±1.5	14.0 mg. ±3.8	12.6 mg. ±2.4	18.0 mg. ±2.7	24.4 mg. ±2.7	28.8 mg. ±1.3	23.0 mg. ±4.0	21.6 mg. ±3.1	7.1 mg. ±3.4	8.3 mg. ±0.9
Thymus Weight	73 mg. ±17	84 mg. ±12	333 mg. ±160	485 mg. ±72	1457 mg. ±152	1738 mg. ±167	1891 mg. ±228	1802 mg. ±430	2055 mg. ± 338	72 mg. ±32	74 mg. ±15
Adrenals Weight	31.0 mg. ±3.3	40.6 mg. ±3.1	44.1 mg. ±3.9	37.0 mg. ±3.2	44.9 mg. ±1.2	49.7 mg. ±2.9	48.7 mg. ±4.4	45.3 mg. ±3.4	46.5 mg. ±2.6	28.9 mg. ±6.2	32.6 mg. ±5.0
Testes Weight	29 mg. ±7	32 mg. ±2	74 mg. ±19	51 mg. ±3	93 mg. ±8	113 mg. ±12	98 mg. ±9	87 mg. ±15	105 mg. ±13	22 mg. ±8	35 mg. ±6
Comb Weight	154 mg. ±48	91 mg. ±16	437 mg. ±18	273 mg. ±26	869 mg. ±182	1463 mg. ±246	1051 mg. ±268	950 mg. ±152	1276 mg. ±282	188 mg. ±119	128 mg. ±24

varied from group to group for several unavoidable reasons. Although the chicks were "sexed," females were found in most groups at the time of autopsy, and these individuals were omitted from consideration. In the low-PGA groups, an occasional chick died before autopsy and likewise there were a few cases of obviously moribund chicks at the time of killing. These were also omitted from the tabulations. The differences between the means of body and organ weights were compared with three times the standard errors[4] of these differences as a measure of significance. In no case was the difference between means of body or organ weights of groups I and II significant. Since the experimental conditions were identical as far as could be determined, these groups were combined in all further considerations. However, the differences between the means of widely separated groups were statistically significant; i.e. groups III and VI with the exception of adrenal weights. Graphic representation (Fig. 1) shows a curvilinear relationship between the level of PGA replacement and body or organ weights. From the table and from graphs, it can be seen that the mean values for the 1000 μg. group were depressed below those of the 200 and/or 80 μg. group with one exception, namely, the pituitary gland. Although these differences were not statistically significant, the consistency of this finding is worthy of note.

An analysis of the organ weights on a body weight basis suggested that the organ weights might be more nearly related to the body weight than to the PGA intake. The coefficients of correlation (r's) were calculated[5] for the various combinations and these statistics are given in Table 2. The growth curves (Fig. 1) showed that there was a marked change in slope between intakes of 80 and 200 μg. of PGA per 100 gm. of food. This was interpreted as meaning that PGA ceased to be the limiting factor for growth at this level. For this reason only the r's of the 0 to 200 μg. groups were compared. Despite the choice of the conditions most favorable to PGA, organ weights appeared to be more closely correlated with body weights than with PGA intake. The mean ratio of hypophyses to body weights and adrenals to body weights progressively decreased as the PGA intake was increased. As can be seen in Table 1, both the hypophyses and adrenals increased in gross weight with increasing PGA supplement.

[4] Calculations were made according to the formulation: The standard error of the difference of two means equals the square root of the sum of the squares of the standard errors of the means.

[5] The coefficients of correlation were calculated by the product moment method according to the formula:

$$r = \frac{\frac{\sum (X - M_x)(Y - M_y)}{N}}{(S.D._x)(S.D._y)}$$

in which X and Y are the individual values in each of the two series considered, M_x and M_y are the arithmetic means of the respective series, and $S.D._x$ and $S.D._y$ are the two standard deviations of the respective means of the two series.

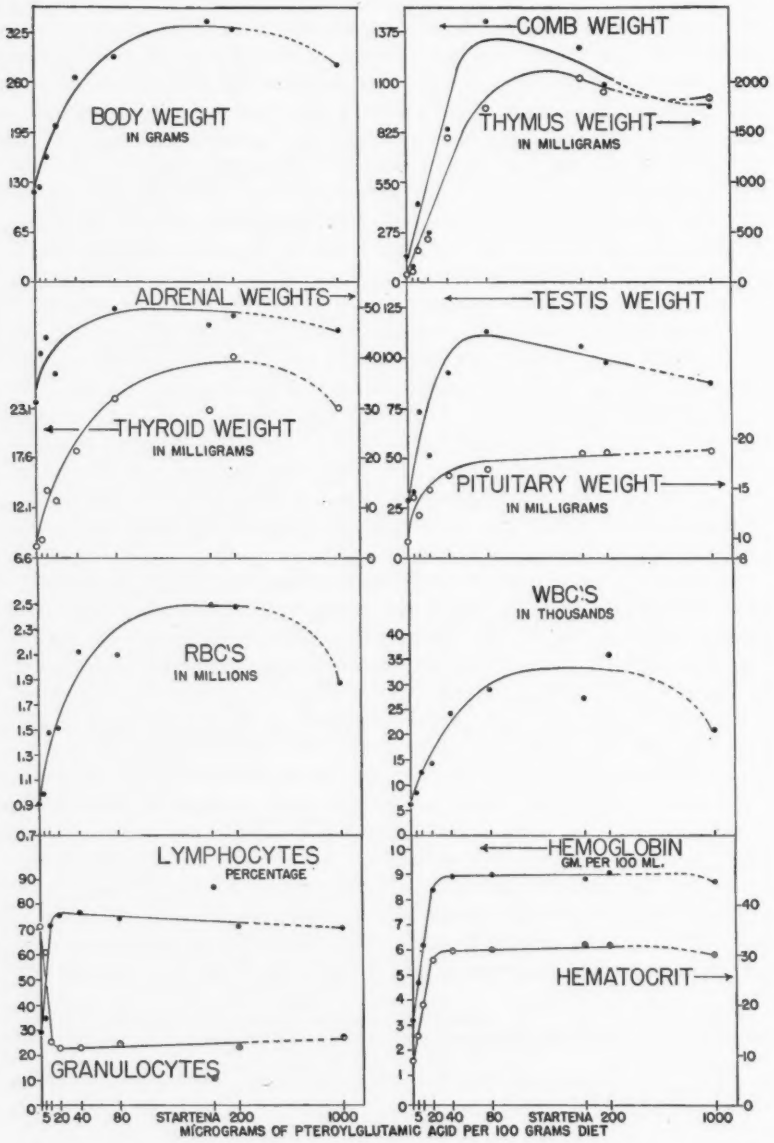


FIG. 1. Curves showing the relations of PGA intake to the body and organ weights, and the various hematologic results. The dotted portions of each curve indicate that the abscissal interval between 200 and 1000 μg. PGA has been shortened to conserve space.

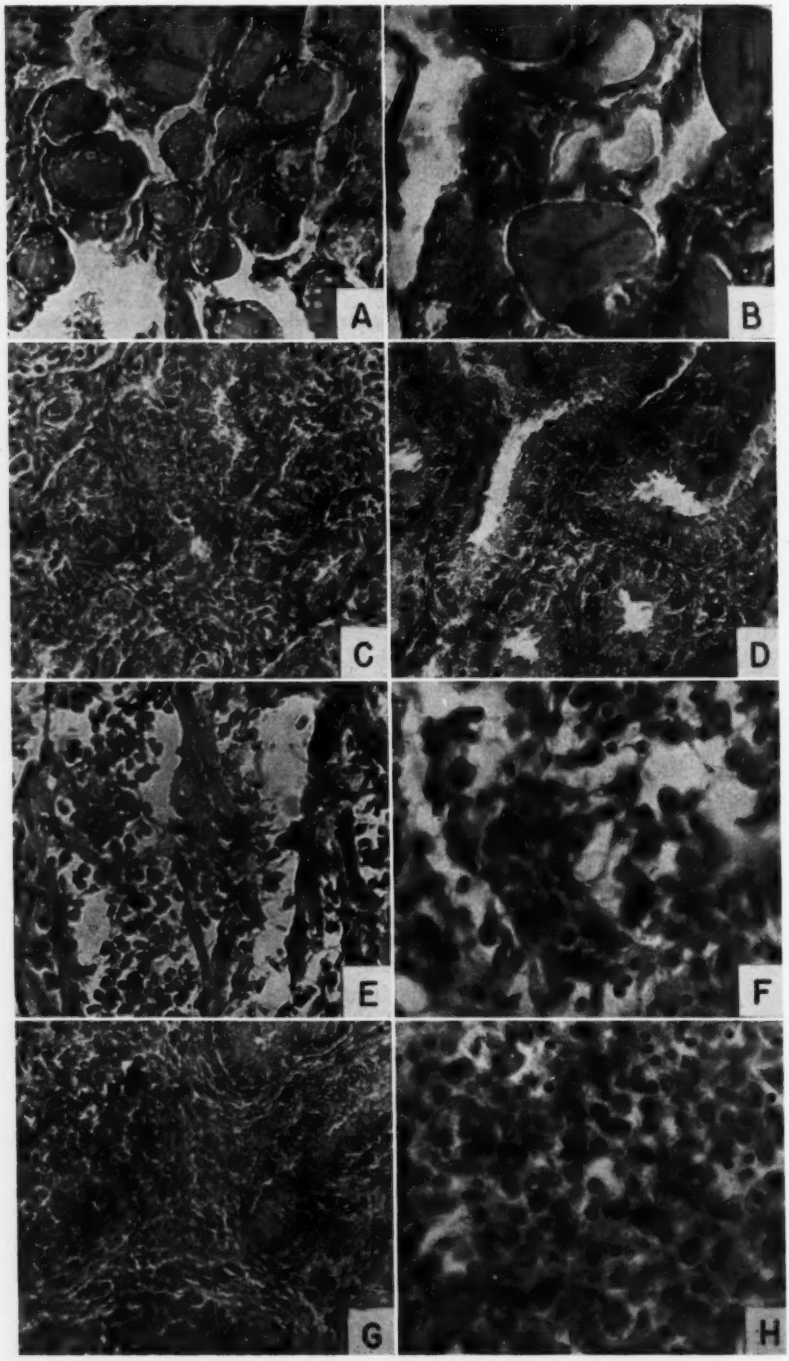
TABLE 2. COEFFICIENTS OF CORRELATION

Organ or result	Coefficients of correlation of 0–200 μg. PGA inclusive	
	With PGA	With body weight
PGA		0.72 ± 0.07
Pituitary	0.55 ± 0.11	0.67 ± 0.09
Thyroid	0.68 ± 0.08	0.84 ± 0.04
Thymus	0.72 ± 0.07	0.91 ± 0.02
Adrenals	0.43 ± 0.12	0.60 ± 0.10
Testes	0.49 ± 0.11	0.80 ± 0.05
Comb	0.51 ± 0.12	0.79 ± 0.06
R B C's	0.68 ± 0.09	0.76 ± 0.06
W B C's	0.77 ± 0.07	0.81 ± 0.11
	Coefficients of correlation of 0–20 μg. PGA inclusive	
Hematocrit	0.72 ± 0.12	0.60 ± 0.16
Hemoglobin	0.92 ± 0.04	0.59 ± 0.18
Lymphocytes	0.79 ± 0.09	0.48 ± 0.18
Granulocytes	0.76 ± 0.10	0.44 ± 0.19

It seems reasonable to interpret these data as indicating that the rate of body weight increase was greater than that of the two organs mentioned. The mean ratio of thymuses to body weights, combs to body weights, and testes to body weights, on the other hand, increased progressively with increased amounts of PGA. This was particularly apparent in the case of the thymuses, and is substantiated by the finding that the coefficient of correlation betweeen the thymuses and body weights was the highest of any calculated. Similar mean ratios calculated for the thyroids failed to fall into any consistent trend and the slope of the curve drawn through the means was relatively insignificant. This may be interpreted to indicate that the physiological activity of the thyroid gland, as reflected by gross organ weights, may be a more important factor in the growth of PGA-deficient chicks than the level of activity of any of the other endocrine glands studied. It should be pointed out that the primary disturbance may be a diminished pituitary activity. No data or observations are at hand to contribute to the solution of this particular problem, however, since the plate-dried pituitary material proved to be insufficient for gonadotrophin and thyrotrophin assays.

Histological Findings.—Organs from representative birds of each group were prepared as described above. The organs were studied microscopically, and only the thyroids, testes and ovaries seemed to show any histological differences between the low- and high-PGA groups. The thymuses and adrenal glands showed no obvious differences in structure or composition, although the differences in gross size were important.

A study of sections of the thyroid glands (Fig. 2, A and B) immediately suggested that PGA deficiency resulted in a retardation of thyroid development. The cells appeared to be smaller, the colloid seemed to be less in amount, there was considerably more interfollicu-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lar tissue, and the colloid seemed to be more vacuolated in the glands of deficient chicks. These glands seemed to resemble those of chronologically much younger individuals. In order to establish this point quantitatively the cell heights in the glands of 3 chicks from groups I and II, IV, VI, and X were measured, with a calibrated ocular micrometer. Four hundred cells from each chick were measured, 100 from each of 4 widely spaced sections. The principles of random sampling were obeyed insofar as it was consciously possible to do so. The same procedure was followed in measuring colloid diameters, but in this case only 20 per section were measured. The data are given below:

Group	Added PGA in μg./100 gm. diet	Cell height in micra	Colloid diameter in micra
I & II	0	4.59±0.12	31.0±2.1
IV	10	4.88±0.16	44.0±3.1
VI	40	6.37±0.18	41.0±2.8
X	Startena (175)	6.62±0.19	46.0±3.2

The testes of groups I and II (Fig. 2, C) were histologically quite variable, but those of groups IV, VI, and X (Fig. 2,D) were rather consistent in appearance. In the latter groups the tubules were composed of tall, columnar cells with basally placed nuclei. Interspersed among these cells were spherical germ cells. Most of the testes of groups I and II contained tubules that seemed to be hyperplastic. The tubular diameters were less than those in the high-PGA groups, but the walls of the tubules consisted of 3 to 4 layers of cells. The constitutent cell types were the same but the cells seemed to be smaller and were more compacted. One bird of these groups was exceptional in that the tubular epithelium was not consistently stratified. The tubular diameter was larger than those of the rest of the groups, and approximated those of the high-PGA groups. Because of the apparent relationship between tubular diameter and PGA intake, measurements were made and these data are presented below. Each average is the arithmetic mean of 600 measurements, 200 for each of 3 birds of each group, plus or minus the standard error.

FIG. 2. A. and B. Photomicrographs of sections of thyroids from, respectively, PGA-deficient and PGA-adequate chicks shown at the same magnifications.

C. and D. Photomicrographs of sections of testes from, respectively, PGA-deficient and PGA-adequate chicks.

E. Section of myocardium of chick from 200 μg. group showing cardiac fibers between which are cells of the hematopoietic type.

F. High power view of section E showing hematopoietic nest.

G. Section of testis of a chick from 80 μg. group showing two sub-capsular areas of hematopoiesis.

H. High power view of section G showing hematopoietic area.

Group	Added PGA in μg./100 gm. diet	Tubular Diameter in micra
I & II	0	39.0±1.6
IV	10	51.0±2.1
VI	40	47.0±1.9
X	Startena (175)	47.0±1.9

Areas of extra-medullary hematopoiesis were found in three different chicks. One chick of group VIII had a large diffuse area in the myocardium (Fig. 2, E and F). Subcapsular nodules (Fig. 2, G and H) were found in the testes of a chick in group VII and a chick in group IX. These areas were at first mistaken for diffuse lymphocytic infiltration, but further study with higher magnification obviated this conclusion. Even in sections stained with hematoxylin and eosin, cells corresponding to primitive "blasts," basophilic erythroblasts, polychromatophilic erythroblasts, and nearly mature erythrocytes were seen. The designation of extra-medullary hematopoiesis was based upon finding these cell types present. The presence of this condition in the high-PGA groups is not interpreted by us at the present time.

Hematological Findings.—The greater part of the hematological data is being presented elsewhere, but it seems advisable to relate some of the findings with PGA intake and/or body weight. In Figure 1 are the curves relating the arithmetic means of the various hematologic data at various levels of dietary PGA. In Table 2 are given the coefficients of correlation between these various statistics and the PGA intake and body weights.

Unlike the correlations of organ weights with body weights the hematocrit, hemoglobin, and lymphocyte and granulocyte percentages show better correlations with PGA intake through the 20 μg. group. The data, therefore, suggest that these hematological determinations should prove to be better criteria in chick assays for this vitamin, so long as the intake is kept below the level of 20 μg. of PGA per 100 gm. On the other hand, body or organ weights should give better assays if the level of intake is above 20 μg. per 100 gm. of diet. It is of interest to note that there is a change in slope of each of the curves relating hematological findings with PGA intake at a comparatively low level of dietary PGA. This may be because the requirements of this vitamin for hematopoiesis may take precedence over the requirements for tissue growth. Alternatively, blood formation may require less PGA for optimal results than does continued body growth.

SUMMARY

The endocrine organ weights of young chicks receiving for 4 weeks a pteroylglutamic acid (PGA)-deficient diet supplemented with various levels of PGA are reported. The data were treated statistically and correlations were made between organ weights and body weights, and

between organ weights and dietary PGA content. In all cases the organ weights were found to be more closely related to body weights than to PGA intake. Similar correlations were made on some of the hematological data obtained from the same chicks. In this case the hematocrit, hemoglobin, and lymphocyte and granulocyte percentages were found to be more closely correlated with dietary PGA than with body weight when the PGA content of the diet was 20 μg. per 100 gm. or less.

Graphic data are presented which show a marked change in slope of the curves relating organ weights and PGA intake at a level of 80–200 μg. per 100 gm. of diet. On the other hand, a similar marked change in slope occurs near the 20 μg. level when hematological findings are considered. The application of these observations to chick assays for PGA is briefly discussed.

A study of histological sections of the endocrine organs indicated that the thyroid glands and testes were most affected. Data are reported for cell heights and colloid diameters of the thyroid glands and for tubular diameters of the testes, indicating that cellular activity is increased directly as the PGA intake is increased.

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OBSERVATIONS ON THE HISTOPHYSIOLOGY OF THE ADRENAL GLAND OF THE GOLDEN HAMSTER

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INTRODUCTION

In recent years a large volume of experimental work on the lipid-rich adrenal glands of the small laboratory animals has demonstrated a close correlation between lipid and ascorbic acid content, and functional activity (Knouff, *et al.*, 1941; Dosne and Dalton, 1941; Flexner and Grollman, 1939; Ludewig and Chanutin, 1947). It appears to have been conclusively demonstrated that physiological stress when first applied produces a fall in the lipid, ascorbic acid, and cholesterol content of these glands. This initial fall is followed during adaptation or recovery from the stress by a rapid restoration of the cholesterol, ascorbic acid, and lipid content. Moreover, Sayers and his colleagues (1944, 1946) have shown that these changes are brought about by variations in the output of adrenocorticotropic hormone.

Since cholesterol and the cortical hormones of the adrenal are both steroids, it has been concluded that the cholesterol is concerned in some still unknown way with the formation of the ketosteroid hormones of the cortex, possibly serving as a precursor (Zwemer, 1936). However, this concept does not take into account the fact that the adrenal cortex in a number of animals is relatively poor in lipid and cholesterol.

The Syrian or golden hamster possesses an adrenal which contains little if any sudanophil lipid (Peczenick, 1944; Koneff, *et al.*, 1946) and no histochemically demonstrable cholesterol. Since it may be assumed that the adrenal of this animal does produce the cortical steroid hormones and since it is "lipid-poor," it was thought that perhaps further insight into the mode of production of these hormones could be gained by comparing the behavior of this adrenal with that of the lipid-rich adrenal in such experimental series as have been designed for the latter.

METHODS AND RESULTS

Commercially obtained male golden hamsters were used throughout this

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study. They were divided into three groups—immature (average initial body weight 55 grams), young adults (average initial body weight 70 grams) used for a preliminary study, and old adults (up to eleven months of age at initiation of experimentation). These groups were then subdivided, according to the treatment to be received, as per Table 1, and treated as described below. At the end of the experimental period for each group, the animals comprising that group, along with the respective controls, were killed by by decapitation. In the preliminary study using the young adults, both adrenals were then quickly excised, weighed, and fixed in either 10% for-

TABLE 1. SHOWING DISTRIBUTION OF HAMSTERS BY GROUPS

Series	Old adults	Young adults	Immature
Desoxycorticosterone acetate	5	10	11
Diethylstilbestrol	7	10	12
Formalin, 4%		2	
Adrenocorticotropic hormone		10	17
Controls	10	12	12

malin or in Heidenhain's Susa fixative (Cowdry, 1948). The latter were cut in paraffin at 5 μ and stained in either Delafield's hematoxylin-eosin or with Masson stain, while the former were cut frozen after 48 hours fixation at 10–15 μ and used for the Schultz cholesterol, sudan orange, or Schiff reactions for carbonyl lipids. Some of the adrenals were also prepared by the method of Da Fano for the study of the Golgi element. The phosphatases (Gomori, 1939, 1941) and phospholipids (Baker, 1946) were studied in some of the control adrenals prepared specifically for those studies. Adrenal weights were converted to the adrenal weight-body weight (A/B) ratio (adrenal weight in mg./body weight in grams $\times$ 100) to correct for variations in body weight. In the later study, involving the immature and old adult animals, the left gland was immediately excised and placed in 10% formalin for at least 48 hours to be used for the sudan orange, Schultz cholesterol, and such pigment studies as were found necessary. The right gland was excised, weighed, ground in 3% metaphosphoric acid, and titrated for ascorbic acid using sodium 2,6-dichlorobenzenone indophenol in a capillary microburette according to the method outlined in Glick (1949). Ascorbic acid concentrations are herein reported as mg./100 mg. of fresh tissue.

Normal: The hamster adrenal is topographically similar to other mammalian adrenals, presenting a cortex which has an outer zona glomerulosa, middle zona fasciculata, and an inner zona reticularis (Fig. 1). Histochemically it differs from those of the common laboratory animals in that there is no reaction with Schultz reagent for cholesterol and its esters nor are there any typical sudanophil lipid droplets. In sudan preparations the whole of the cortex is tinted a diffuse yellowish orange which is quite unlike the coloring produced by lipid-filled vacuoles. This peculiar coloring of the hamster adrenal may be due to lipid-rich mitochondria. The only lipid substances we did find were the phospholipids visualized by Baker's method and the carbonyl lipids demonstrated by Schiff's reagent after 48 hours of

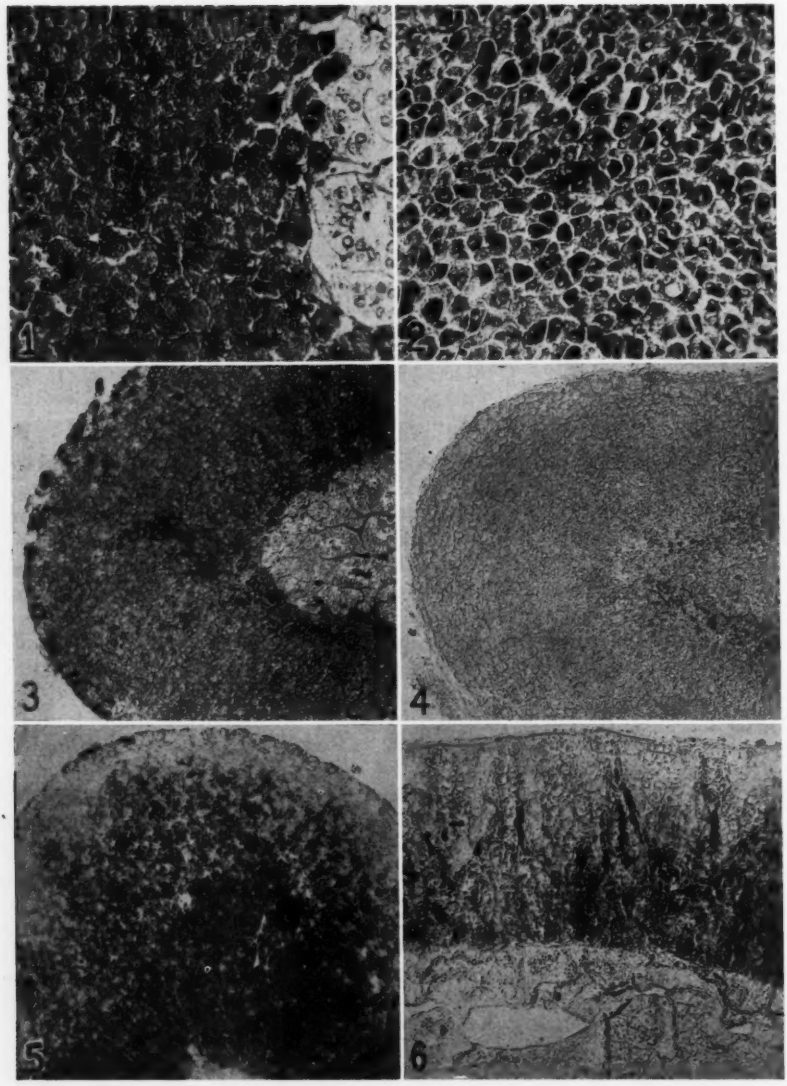

FIG. 1. General topography of normal male hamster adrenal showing inner portion of cortex and part of the medulla. Note relative compactness of cortical cells and compare with Figure 2. Masson stain.

FIG. 2. Cortex of young male hamster adrenal treated with diethylstilbestrol. Note engorgement of blood sinuses. Masson stain.

FIG. 3. Adrenal of old male hamster treated with diethylstilbestrol. Note sudano-

formalin fixation. The former were found as fine black cytoplasmic granules throughout the cortex. The carbonyl lipids occupy the whole of the cortex, producing an intense stain with Schiff reagent, the color being limited to the cytoplasm. These carbonyl lipids react directly with Schiff reagent, having already been unmasked by the formalin. The alkaline phosphatases are predominantly located in the cells of the outer half or two-thirds of the zona fasciculata in both nuclei and cytoplasm; only the nuclei of the other regions are but faintly tinted (black). Chemical analysis of the right adrenal shows it to contain from .114 to .172 mg. of ascorbic acid per 100 mg. of fresh tissue (Tables 4 and 5). The cortical Golgi apparatus is similar to that described by Bourne (1934) and Hoerr (1936).

Desoxycorticosterone acetate: This hormone was administered every other day subcutaneously in a 0.85% saline suspension containing 5

TABLE 2. AVERAGE ADRENAL WEIGHT-BODY WEIGHT RATIOS FOR SEVERAL GROUPS OF MALE HAMSTERS

Series		Old adults*	Young adults	Immature*
Desoxycorticosterone acetate	Experimental	7.6	17.1	9.7
	Control	8.3	19.0	10.3
	Difference	−8.4%	−10%	−5.8%
Diethylstilbestrol	Experimental	6.3	17.1	8.4
	Control	8.3	19.0	10.3
	Difference	−24.1%	−10%	−18.4%
Formalin stress	Experimental		26.0	
	Control		19.0	
	Difference		+36.8%	

* A/B ratio for right adrenal only.

mg. of hormone per cc., the volume of each injection. The immature animals each received a total of 80 mg. of hormone and the adults each received a total of 105 mg. during the course of the experiment. Upon sacrifice, it was found that the adrenals showed a slight decrease in weight (Table 2). Microscopically, the zona glomerulosa appeared thinner, possibly indicating a reduced rate of cell replacement. No other histological changes were apparent. The rate of body weight gain in these animals during the course of the experiment was approximately 60% that of the control group. Histochemically these adrenals showed no change in the diffuse "background wash" stain with sudan orange or black. The Schultz cholesterol test was consistently negative. Changes were noted, however, in the carbonyl lipids. The

philia (black) due to lipochrome pigment in the zona reticularis and extending into the zona fasciculata. Sudan orange.

FIG. 4. Unstained frozen section of same gland as in Figure 3. Note pigment in the zona reticularis (black).

FIG. 5. Adrenal of old male hamster treated with diethylstilbestrol. Note heavy sudanophilia. Sudan orange.

FIG. 6. Same gland as in Figure 5. Schultz reaction. Note heavy amounts of cholesterol radiating into the zona fasciculata from the zona reticularis.

TABLE 3. AVERAGE ADRENAL WEIGHT-BODY WEIGHT RATIOS OF SEVERAL GROUPS OF IMMATURE MALE HAMSTERS TREATED WITH ADRENOCORTICOTROPIC HORMONE

Series	Elapsed time between last injection and sacrifice	A/B ratios	Control	Difference
1 injection of 1 mg.* per day for 10 days	24 hours	24 (10)†	19 (12)	+26.3%
1 injection of 2 mg.	1 hour	10.2(4)‡	11.2(9)	− 9%
	12 hours	12.8(3)‡		+14%
	24 hours	12.3(3)‡		+10%
3 injections of 2 mg. each per day for 3 days	3 hours	16.7(4)‡		+49%
	38 hours	14.7(3)‡		+31%

* In each case, the injection volume was 0.5 cc., pH of suspension was 7.0.
† Ratio for both adrenals
‡ Ratios for right adrenal only. Numbers in parentheses refer to number of animals in series.

outer zona fasciculata and zona glomerulosa only were stained, leaving unstained the rest of the gland. This was the exact opposite of what has been reported in the rat (Sarason, 1943; Greep and Deane, 1947). Chemically, the ascorbic acid was depleted by as much as 34% in the old adult animals (Table 4).

Diethylstilbestrol: The young adults used in the preliminary survey each received daily one subcutaneous injection of one cc. of a solution of sesame oil containing 1 mg. of hormone, to receive a total of 27 mg. during the course of the experiment. The immature and old adult animals each had one 25 mg. pellet implanted subcutaneously to act over periods of 3 months and 4 months respectively. The rate of body weight gain for the latter two groups was 58% that of the normal. The adrenals suffered weight losses depending upon the length of exposure to the hormone (24% in the case of the old adults) (Table 2). Microscopically, the cortical blood sinuses were seen to be dilated (Fig. 2), although the cells of each of the zones appeared normal in size, possibly indicating a loss in the total number of cortical cells. This was the only apparent morphological change. The chemical analysis showed a loss in adrenal ascorbic acid of 11.8% in the case of the old adults but a gain of 36% in the immature animals (Table 4). An

TABLE 4. EFFECTS OF DESOXYCORTICOSTERONE ACETATE AND DIETHYLSTILBESTROL ON THE ASCORBIC ACID OF THE RIGHT ADRENAL*

Series		Old adults	Immature
Desoxycorticosterone acetate	Experimental	.106	.107
	Control	.161	.114
	Difference	−34.2%	−6.1%
Diethylstilbestrol	Experimental	.142	.155
	Control	.161	.114
	Difference	−11.8%	+36%

* Reported as mg./100 mg. fresh tissue

important finding in the adrenals of the animals treated with estrogen was a sudanophil pigment in the zona reticularis (Fig. 3) which radiated out into the zona fasciculata for varying distances. This pigment was observable in the freshly cut unstained frozen section (Fig. 4) and was not extracted by alcohol or acetone. Histochemically, it gave no reaction with either concentrated sulfuric acid or with iodine (Lugol's), establishing its lipochrome nature as contrasted with the carotinoids (Lison, 1936). This is the same pigment seen in "brown degeneration" of various organs of old animals and also found in mice treated with estrogen by Cramer and Horning (1937) and Tobin and Birnbaum (1947). The pigment was present in greatest amounts in those animals (old adults) which had the longest exposure to the hormone (four months). All but one of these animals exhibited the pigment, as did about one-fourth of controls. Pigment was observed in half of the animals which were treated for only three months (the immature animals). The controls of this group did not have any adrenal pigmentation. Apparently diethylstilbestrol has degenerative effects upon the adrenal cortex. There was no pigmentation found in any of the adrenals of the young adult group which received the hormone-in-oil injections for one month. The one old adult which did not exhibit any adrenal pigmentation showed instead a marked sudanophilia radiating out from the zona reticularis to include most of zona fasciculata (Fig. 5). Upon treatment with Schultz reagent, a heavy cholesterol concentration was found (Fig. 6), as evidenced by the green coloration, closely approximating the sudanophil area in distribution. Both the sudanophilic and Schultz reactions were negative after treatment of the sections with alcohol-ether. In other species, diethylstilbestrol has resulted in an increase in lipid containing vacuoles (Allen and Bern, 1942), presumably due to its action upon the anterior hypophysis to produce adrenocorticotropic hormone (Levin, 1945). Although this will raise the cholesterol content of the rat and guinea pig adrenals above normal (Sayers, *et al.*, 1944, 1946), this is the first reported instance, to this author's knowledge, in which cholesterol has ever been found in the hamster adrenal. The only other histochemical change noted was reduction in the carbonyl lipids, mainly in the inner half of the cortex.

Adrenocorticotropic hormone: This hormone was administered to two different groups of animals. The young adults each received daily for ten days one intraperitoneal injection of a suspension in 0.85% saline containing 1 mg. per cc., the volume of each injection. The pH of the suspension was adjusted to 7.0. The adrenals of these animals showed a 26% gain in weight (Table 3). Histochemically, there was an evenly distributed reduction in carbonyl lipids. As a control measure, two hamsters of the same age and weight were given four injections in two days of 0.5 cc. of a 4% solution of formalin to effect the "stress reaction" (Selye, 1937). The carbonyl lipid picture here was

identical to that just described above and the weight was also increased (Table 2). No other changes were noted in either group. The second group of experimental animals, all immature, were treated according to the method of Sayers, *et al.*, (1946) to determine the behavior of the hamster adrenal ascorbic acid with respect to time and dosage. The results were quite different from those reported by Sayers in the rat and the guinea pig. The weights were not markedly affected after one injection but whereas in the rat and guinea pig the greatest weight increment was found 38 hours after the last of nine

TABLE 5. EFFECT OF ADRENOCORTICOTROPIC HORMONE ON THE ASCORBIC ACID OF THE RIGHT ADRENAL OF IMMATURE MALE HAMSTERS

Series	Elapsed time between last injection and sacrifice	Ascorbic acid (mg./100 mg. fresh tissue)		
		Experimental	Control	Difference
1 injection of 2 mg. (0.5 cc.)	1 hour	.162(4)*	.172(9)	− 6%
	12 hours	.174(3)		± 0%
	24 hours	.149(3)		−13%
3 injections of 2 mg. (0.5 cc.) each per day for 3 days	3 hours	.168(4)		± 0%
	38 hours	.125(3)		−26%

* Numbers in parentheses refer to number of animals in series.

injections in three days, in the hamster the greatest weight increment (49%) occurred after three hours (Table 3). The ascorbic acid differences were even greater, first decreasing, returning to normal, and finally rising above normal in the rat and in the guinea pig, but decreasing, returning to normal and then again decreasing in the hamster (Table 5). Neither the weight gains nor the ascorbic acid losses were as marked in the hamster as they were in the rat and the guinea pig as reported by Sayers. The potency of the hormone used in these experiments was tested on adult male rats by injecting one cc. of 0.85% saline suspension containing four mg., intraperitoneally. Upon sacrifice three hours later, it was found that the adrenal ascorbic acid had been depleted by 65%, thus removing any doubt as to the potency of the hormone. Apparently the hamster adrenal is not as easily stimulated by adrenocorticotropic hormone as are the adrenals of the rat and guinea pig. Furthermore, as shown above, the hamster adrenal behaves in a different manner than the rat and guinea pig adrenals under the influence of the hormone.

DISCUSSION

The hamster possesses an adrenal of the lipid-poor type. It therefore appeared highly desirable to perform the same experiments on this animal that have been performed on animals with lipid rich adrenals. Koneff, *et al.*, (1946) report that chronic administration of diethylstilbestrol produces an appearance of considerable amounts of

sudanophil material in both the male and female hamster adrenal. Since they did not employ the Schultz cholesterol test no conclusions regarding cholesterol may be drawn from their results. The sudanophil material appeared in the inner zones of the cortex and spread outward.

In our experiments described above, we likewise achieved a sudanophilia but this was due in all but one instance to degeneration pigment and in only one to cholesterol. A slight reduction in adrenal weight was observed, which was also reported by Koneff, *et al.* This is especially significant since many investigators (Leathem & Silverman, 1945; James and Nelson, 1942; von Haam, *et al.*, 1941) have reported an increase in adrenal weight following such treatment. Levin (1945) concluded that this substance had its effect on the adrenal via the anterior pituitary, that is, a stress reaction. However, it should be pointed out that diethylstilbestrol does have at least a partial pituitary inhibitory effect—on the gonadotropic hormones. The testes of all our animals in this series were markedly atrophied, a finding also reported by Nalbandov and Baum (1948) in the rat.

The animals treated with desoxycorticosterone acetate presented adrenals which showed slight losses in weight as has been reported for other animals (Sarason, 1943; Selye and Hall, 1943; Selye, 1940a; Selye 1940b; Selye and Dosne, 1940; Greep and Deane, 1947; Carnes, *et al.*, 1941). This effect is presumably due, in part, to the inhibitory effect of the hormone upon the anterior pituitary to cut down on its secretion of adrenocorticotropic hormone. This fact, however, does not account for the marked loss of adrenal ascorbic acid (34%) in the case of the older animals. According to del Castillo and Rapela (1945), desoxycorticosterone acetate may also have a direct effect upon the adrenal cortex. This might explain the reduction in adrenal ascorbic acid. Since the behavior of adrenal ascorbic acid so closely parallels that of adrenal cholesterol (Sayers, *et al.*, 1946), it might be inferred or expected that the ascorbic acid would remain at relatively the normal level. Another variation in the hamster is a decrease in the rate of body growth rather than the increase over the normal that has been reported in other species. The carbonyl lipids likewise behave differently in the hamster treated with desoxycorticosterone acetate. Whereas in the rat this hormone depletes the glomerulosa, in the hamster it was the inner fasciculata and reticularis that were depleted of their Schiff-stainable material. Obviously, the hypothesis that has been advanced concerning the mode of function of this hormone in other animals (Greep and Deane, 1947) does not hold for the hamster. This is likewise true of diethylstilbestrol.

The adrenocorticotropic hormone groups showed changes similar to other species in that there was a generalized adrenal weight gain, but the rates of gain at various times following one or more injections differed from that of the rat and guinea pig. At the time when other animal adrenals had reached their maximum weight gain, the hamster

adrenal was already receding to a normal weight. This fact, along with the fact that the adrenal ascorbic acid did not behave like that in the rat or guinea pig, show that the hamster adrenal also reacts differently with adrenocorticotropic hormone, at least in some respects.

It is obvious from the foregoing that while the hamster adrenal is topographically similar to other mammalian adrenals, its histophysiology resembles those of other mammals to but a very slight extent. The only lipids that we have been able to demonstrate in the normal male hamster adrenal by histochemical methods are the Schiff-positive lipids and the phospholipids visualized by the method of Baker. We were unable to determine the content of this phospholipid material in our experimental animals because of a lack of material. The only lipid change noted in our experimental animals was found in the carbonyl lipid content, it being decreased in every instance. From these results the conclusions may be made that in the hamster adrenal, which is of the lipid poor type, cortical hormone production is correlated with this Schiff-positive (carbonyl) lipid.

As stated previously, the chemical nature of this Schiff-positive material is uncertain. It was termed plasmal by Feulgen and Voit (1924), who devised the test. The test was originally applied to fresh, unfixed frozen sections of the adrenal and was shown by Feulgen and Bersin (1939) and others that it demonstrates acetal phospholipids or plasmalogens. Dempsey and Wislocki (1946), have however, modified this test by applying the Schiff reagent to formalin-fixed sections and claim that it demonstrates ketosteroids, basing this claim on the work of Bennett (1939, 1940) and Gomori (1942). The substance(s) demonstrated by our material are, in any event, not known to be plasmalogens since the action of mercuric chloride had no effect whatever on the result (Hayes, 1949). On the other hand, it has been reported that ketosteroids do not react at all with the Schiff reagent (Ashbel and Seligman, 1949).

In any event, our findings that physiological stress and adrenocorticotropic hormone stimulation produce a decrease in the Schiff-positive lipid after formalin fixation support the view of Dempsey and Wislocki that the Schiff positive material demonstrated after formalin fixation is correlated with adrenocortical activity but the conclusion that this material is actually hormone substance is questionable. These results in a lipid-poor adrenal also indicate that the sudanophil and cholesterol changes associated with cortical activity in the lipid-rich adrenals are secondary and incidental changes and suggest that although these changes register the activity of the gland, the cholesterol material is either (1) not a precursor but a by-product of hormone production in lipid-rich adrenals and is not produced at all in lipid-poor adrenals, or (2) it may be that the cholesterol is a precursor of hormone production but that in the hamster the cholesterol is utilized immediately for hormone production and so never reaches histochemically demonstrable (0.5%) concentrations.

It would appear from the differences in histophysiology noted between the adrenal of the hamster, a lipid-poor adrenal, and those of the other small laboratory animals, of the lipid-rich type, that the results derived from an experimental series on one animal do not necessarily apply to any other animal and theories regarding function should be formulated only from the results obtained from many, if not all, types of animals.

SUMMARY

The adrenal cortex of the normal male hamster is topographically similar to that of other mammalian adrenals since it presents a fairly well defined zona glomerulosa, zona fasciculata, and zona reticularis. It differs from the adrenals of other common laboratory animals in respect to lipid content. It contains no Schultz-positive cholesterol nor typical sudanophil lipid.

Injections of desoxycorticosterone acetate produced a slight reduction in adrenal weight but no evident change histologically. The only histochemical change produced was the disappearance of carbonyl lipid from the zona reticularis. Diethylstilbestrol produced a slight reduction in adrenal weight. This was accompanied by vascular engorgement which indicates some cortical cell atrophy. Cortical atrophy, however, was not apparent histologically. The appearance of sudanophil material following treatment with estrogen as reported by Koneff, *et al.*, (1946) was confirmed, but in this case was shown to be due to degeneration pigments or lipochromes. The finding of cholesterol and lipid sudanophilia in one instance is reported. The administration of adrenocorticotropic hormone produced a definite increase in adrenal weight. The carbonyl lipids were very markedly reduced, no other histochemical change being evident. Stress likewise produced a definite increase in adrenal weight and a marked reduction in carbonyl lipid. No other changes were noted. Chemical analyses for adrenal ascorbic acid showed this substance to be affected to the greatest extent by diethylstilbestrol and adrenocorticotropic hormone. However, the latter substance did not affect the adrenal ascorbic acid of the hamster as it did that of the rat and guinea pig as reported by Sayers, *et al.*, (1946).

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THE DIFFERENTIAL MECHANISMS BY WHICH PICROTOXIN AND COPPER ACETATE INDUCE OVULATION IN THE RABBIT

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INTRODUCTION

OVULATION, in species like the rabbit that do not ovulate spontaneously, is normally induced via a neurohumoral reflex initiated at coitus. The afferent pathways in the reflex arc are poorly understood, but the efferent route almost certainly involves the hypophyseal stalk (Brooks, 1937, 1938). Electrical stimulation experiments (Markee, Sawyer and Hollinshead, 1946; Harris, 1948b) favored a vascular pathway, such as the hypophyseal portal system (Wislocki and King, 1936; Green and Harris, 1947), over direct nerve fibers (Brooks, 1940) as the route by which the hypothalamus exerts its control over the anterior hypophysis. Markee, Sawyer and Hollinshead (1948) found that the injection of small amounts of epinephrine directly into the adenohypophysis activated the release of LH. That an adrenergic mediator is involved in the natural secretion stimulus was affirmed by blocking copulation-induced ovulation with the adrenergic blocking agent Dibenamine (Sawyer, Markee and Hollinshead, 1947). Subsequent work provided indirect evidence that this reflex includes a cholinergic (atropine-blocked) component which precedes and may initiate the adrenergic component (Sawyer, Markee and Townsend, 1949). Nickerson (1949) has recently criticized the interpretation of the Dibenamine results, but these objections have been answered (Sawyer, Markee and Everett, 1949, 1950). The importance of the nervous system in the control of ovulation in species other than those requiring a coital stimulus has been emphasized by Sawyer, Everett and Markee (1949), and Everett, Sawyer and Markee (1949) who found that adrenolytic and anti-cholinergic agents, and, recently (Everett and Sawyer, 1949), anesthetic drugs in appropriately timed injections, would block ovulation in the rat.

In view of this newer information it seemed desirable to reinspect the mechanisms by which chemical agents such as picrotoxin and copper acetate induce ovulation. Marshal, Verney and Vogt (1939) found that, among several central excitatory drugs, picrotoxin alone was capable of producing ovulation in the rabbit. Since all but one of

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the ovulating animals had experienced severe convulsions there was little reason to doubt that the drug stimulated the release of LH indirectly via the nervous system. A reduced dose of picrotoxin gave near-lethal convulsions in the atropinized rabbit, but such treatment was not followed by ovulation. This failure might indicate either that atropine blocks pituitary activation or merely that the picrotoxin dosage was too low. Brooks *et al.* (1940) furthered the hypothesis that picrotoxin stimulates the pituitary by way of the nervous system by demonstrating that picrotoxin fails to induce ovulation in rabbits whose pituitary stalks have been severed. Recently Kasdon (1949) has had considerably less success in stimulating ovulation with picrotoxin; only 4 out of 27 ovulated in spite of the fact that 21 were postpartum animals. Kasdon attempted to demonstrate, employing phenobarbital to protect his rabbits from death, that the capacity of picrotoxin to induce ovulation is independent of dosage. It will be shown below, however, that barbiturates block the ability of picrotoxin to stimulate LH release. Like picrotoxin, metrazol has been employed successfully to stimulate the release of LH in the normal estrous rabbit (Brooks *et al.*, 1940) but not in the stalk-sectioned animal.

Also widely accepted has been the idea that copper salts stimulate the nervous system primarily and the adenohypophysis only indirectly. Fevold, Hisaw and Greep (1936), who discovered that copper salts would induce ovulation in the rabbit, proposed that the effect was one of synergism between copper and the circulating gonadotrophic hormones. Bischoff (1938) tested the synergism hypothesis with negative results and suggested that copper acetate stimulated the nervous system by virtue of its toxicity. Brooks *et al.* (1940) reported the confirmatory negative evidence that section of the stalk blocked copper-induced ovulation. Harris (1941) presented positive results which appeared to confirm the nervous-stimulating role of copper: injections of copper acetate in doses as low as 1/200 the effective systemic level, directly into the third ventricle, induced LH release in 10 out of 13 estrous rabbits. Emmens (1940) found that various salts of copper and cadmium would produce ovulation, but his experiments were not designed to differentiate between a direct effect on the gland and an indirect neural stimulus. Dury and Bradbury (1943) demonstrated that preliminary treatment with estrogen facilitated copper-induced ovulation in anestrous rabbits, and Bradbury (1944b) showed that when ovulation was successfully induced by copper the animal's pituitary gonadotrophin had been discharged. Ovulation following the injection of copper has been blocked by antigonadotrophic serum which prevents the action of the released pituitary hormone (Emmens, 1940), and by progesterone (Friedman, 1941). Copper salts have also been shown to induce pseudopregnancy in the estrous or estrogen-treated rat (Dury and Bradbury, 1942, 1943), but picrotoxin has been reported to be ineffective in this capacity (Ubatuba, 1947). In a small

number of constant estrous guinea pigs, Dey (1943) was unable to induce ovulation with copper acetate whether injected systemically, intrahypophyseally or into the third ventricle.

The present work, which re-examines the mechanisms by which picrotoxin and copper acetate induce ovulation in the rabbit, employs estrogen to facilitate the LH release. The blocking agents of nervous activity, Dibenamine, atropine and Nembutal are used to differentiate between direct and neurally transmitted effects. Picrotoxin is seen to act by way of the nervous system: only rabbits with convulsions subsequently ovulated, and LH release was blocked by Nembutal, Dibenamine and atropine. However, copper acetate consistently gave indications of a direct action on the adenohypophysis: convulsions were absent following copper injections and the copper stimulus was effective in spite of either Nembutal, Dibenamine or atropine. Furthermore, on the positive side, ovulation was induced by injecting tiny amounts of copper acetate directly into the adenohypophysis. Thus a fundamental difference is indicated between picrotoxin and copper as to their sites of action and their mechanisms of stimulation. Part of the results of the present study have appeared in a preliminary report (Markee and Sawyer, 1949).

MATERIAL AND METHODS

Fifty-one female rabbits were employed in the picrotoxin experiments, and 104 received copper acetate. Many were used more than once, and the total number of experiments exceeded 200. The weights of those used in experiments on ovulation ranged from 2.1–4.3 kg. with an average of 2.9 kg., and only 3 of these rabbits weighed less than 2.4 kg. The animals were usually isolated for 2 or more weeks before treatment but some were employed after only a few days isolation. Laparotomies or autopsies were performed routinely at 48 hours and never longer than 72 hours after treatment so it was possible to exclude from the experiments those animals of the brief isolation group that had become pregnant or pseudopregnant before treatment. After laparotomy each rabbit was again isolated for 3 weeks prior to further treatment if pseudopregnant or 2 weeks if it had failed to ovulate.

Our source of the copper ion was the mixture of copper acetate and sodium acetate first employed by Dury and Bradbury (1942). Two per cent solutions of Merck's reagent grade cupric acetate and sodium acetate were made up, stored in the refrigerator and mixed in equal parts just before use. The routine intravenous dosage was 1 ml. or 10 mg. copper acetate, which is the minimal dosage of this salt which produces ovulation in estrous rabbits (Emmens, 1940; Dury and Bradbury, 1942). For intrahypophyseal injections the mixture was diluted to 0.1% with distilled water. The picrotoxin solution was 0.3% in isotonic sodium chloride with 1.5% benzyl alchol as a preservative. The Nembutal (pentobarbital sodium) solution was 6% in water containing 10% alcohol and 20% propylene glycol. Dibenamine (N, N-dibenzyl-β-chloroethylamine) and atropine sulfate solutions were made up daily as in the earlier study (Sawyer, Markee and Townsend, 1949). Dibenamine was used in 1% solution; atropine sulfate in 3% solution for intravenous injec-

tions and 30% for subcutaneous injections, all in unbuffered Ringer-Locke solution.

The estrogen used in these experiments was α-estradiol benzoate dissolved in peanut oil at a concentration of 0.85 mg./ml. After the first series of experiments had confirmed its value in facilitating copper-induced ovulation, all subsequent rabbits received daily subcutaneous injection of 85 μg. estrogen for two successive days prior to copper or picrotoxin administration. A large number of the animals was not naturally in heat since most of these experiments were conducted during the months of October through January.

The operative approach to the hypophysis for intrahypophyseal injection was by way of the parapharyngeal route (Markee *et al.* 1946 1948). With a rubber breathing tube threaded through the mouth into the trachea to prevent collapse of the pharynx, an attempt was made with a probe to break a small hole through the bony base of the sella, exposing the membrane which includes the periosteum, dura and hypophyseal membrane (see Wislocki, 1937, for meningeal relationships). Four attempts were made at 5 or 10 minute intervals to inject 35–40 μl. 0.1% copper acetate through the membrane into the adenohypophysis, the total dosage being about 0.15 ml. or 0.15 mg. copper acetate. Twenty-five rabbits were operated on in this manner, and 20 survived for 48 hours. In 8 animals, all of which survived, India ink was added to the copper solution to aid in tracing the path of the injected medium. At 48 hours the animals were killed by air embolus, perfused through the heart with physiological saline to drive off the blood, and a careful dissection of the sella and the base of the brain was made to determine, by the location of the inflicted wound or the distribution of the India ink, the exact site of the injection. If the hypophyseal capsule was found to have been perforated, the entire hypophysis including the stalk and median eminence was fixed in Zenker's solution, sectioned sagittally in paraffin at 10 μ and stained with acid fuchsin or Harris' hematoxylin and a modification of Mallory's tri-acid stain (Everett, 1943).

Also fixed for histological study were the ovaries in the intrahypophyseal injection series and those of other series removed at laparotomy; ovaries were removed only if they had hemorrhagic follicles or other indications of partial activation or if there was any doubt about whether a follicle had ruptured. Hemorrhagic follicles were considered as positive evidence of pituitary activation only if histological study revealed new luteinization of such follicles.

RESULTS

Physiological responses to injections of the stimulating and blocking agents. No noticeable neurological symptoms followed the injection of the routine dosage of buffered copper acetate-sodium acetate solution. This dose of 10 mg. copper acetate per rabbit was of the order of 15% of the lethal dosage—50–70 mg. (Dury and Bradbury, 1942). The observed physiological effects of Dibenamine and atropine in the rabbit have been described previously (Sawyer *et al.*, 1947, 1949). The injection of copper acetate before or after these blocking agents appeared to alter their physiological effects not at all. Nor did copper seem to exert any influence on the depth or duration of Nembutal anesthesia.

Picrotoxin, on the other hand, in dosages of 1.5–2.0 mg./kg. usually induced severe convulsions after a latent period of 5–15 minutes from the time of intravenous injection. In 3 rabbits the convulsions were allowed to run their course, and the animals died. Thereafter, a quieting dose of Nembutal (30 mg. per animal, about 10 mg./kg.) was injected after the convulsions had proceeded for a very few minutes. Five animals receiving 1–1.5 mg./kg. picrotoxin did not suffer convulsions though they did show hyperexcitability. After an anesthetic dose of Nembutal (40 mg./kg.) no convulsions followed an amount of picrotoxin (4 mg./kg.) which would certainly otherwise have been lethal. Following atropine or Dibenamine, however, very severe and frequently lethal convulsions were induced by dosages of picrotoxin often so reduced that alone they could scarcely have caused even hyperexcitability. Thus, as far as convulsions are concerned, atropine and Dibenamine are synergistic with, and Nembutal antagonistic to, picrotoxin.

TABLE 1. EFFECT OF PRETREATMENT WITH ESTROGEN ON PITUITARY ACTIVATION BY COPPER ACETATE

Number of rabbits	Condition prior to treatment	Estrogen μg.	Copper acetate mg.	Results		
				Activation of hypophysis (ovulation or hemorrhagic follicles	Failed to activate	Percentage activated
8	Estrous	2 (85)	—	0	8	0
8	Anestrous	2 (85)	—	0	8	0
5	Estrous	—	10	5	0	100
5	Estrous	2 (85)	10	5	0	100
7	Anestrous	—	10	0	7	0
7	Anestrous	2 (85)	10	7	0	100

Effects of estrogen pretreatment on the stimulation of ovulation by copper. The results are summarized in Table 1. Estrogen alone did not stimulate the release of luteinizing hormone even in the rabbits that were apparently in heat. Copper was effective without estrogen treatment in producing ovulation in 5 estrous rabbits, and 5 other estrous females responded positively to copper after estrogen pretreatment. The experiment shows that although estrogen injection is not required for ovulation in estrous animals, the preliminary administration of estrogen to this group is not inhibitory to copper activation of the hypophysis. On the other hand, none of 7 anestrous rabbits without estrogen was stimulated by copper, whereas after estrogen, copper induced ovulation in each of 7 initially anestrous animals. The results confirm the finding of Dury and Bradbury (1943) that estrogen facilitates copper-induced ovulation. Because of this evidence and our observations that estrogen facilitates neurogenic stimulation of ovulation in the rat (Sawyer, Everett and Markee, 1949) and reflexogenic stimulation of ovulation in the rabbit (Sawyer, 1949), we have treated

all subsequent animals, reported in this study, with two daily injections of estrogen prior to administration of copper or picrotoxin.

Experiments with picrotoxin and blocking agents. The results of experiments with picrotoxin and blocking agents, such as are concerned with convulsions and pituitary activation, are summarized in Table 2. The convulsions have already received comment. The results of lethal convulsions are included in Table 2 as evidence of the synergistic actions of Dibenamine and atropine with picrotoxin, and to indicate the difficulties of augmenting the survival series. It can be seen that of the surviving animals treated with picrotoxin alone none ovulated

TABLE 2. EFFECTS OF BLOCKING AGENTS AND OF CONVULSIONS ON PICROTOXIN-INDUCED OVULATION IN ESTROGEN-TREATED RABBITS. ATROPINE AND DIBENAMINE WERE WERE INJECTED ABOUT AN HOUR, AND NEMBUTAL ONE MINUTE, BEFORE PICROTOXIN

Number of animals	Blocking agent			Picrotoxin dose range mg./kg.	Convulsions	Results		
	Type	Dosage mg./kg.	Route			Activation of hypophysis (ovulation or hemorrhagic follicles)	Failed to activate	Percentage activated
3	—	—	—	2.0	+(Lethal)	—	—	—
12	—	—	—	1.5 –2.0	+	6	6	50
5	—	—	—	1.0 –1.5	−	0	5	0
6	Diabenamine	30	i.v.	0.75–1.5*	+(Lethal)	—	—	—
5	Dibenamine	30	i.v.	0.75–1.25*	+	0	5	0
5	Atropine	300	sub. cut.	0.5 –1.5*	+(Lethal)	—	—	—
5	Atropine	300	sub. cut.	0.5– 1.0*	+	1	4	20
7	Nembutal	40	i.v.	4.0	−	0	7	0

* The usual dose was the smaller value.

in the absence of convulsions and only half of those that suffered convulsions revealed pituitary activation. It should be remembered that most of the latter group, including 5 of the 6 that ovulated, received quieting doses of Nembutal after about two minutes of severe convulsive seizures. Of the surviving rabbits injected with Dibenamine or atropine prior to picrotoxin administration, only one ovulated in spite of convulsions which were even more severe than those induced by picrotoxin alone. It should be pointed out that the dosage of picrotoxin in these series was only about half as great as that employed in the absence of the blocking agents. Therefore, certain assumptions, to be discussed later in the paper, must be made if Dibenamine and atropine are to be considered as truly blocking agents of picrotoxin-induced ovulation. Nembutal, in contrast, blocks picrotoxin-activation of the hypophysis in a manner parallel to its inhibition of picrotoxin convulsions, even when the picrotoxin dosage has been elevated. The results are consistent with the hypothesis that picrotoxin activates the hypophysis by its action on the central nervous system.

Experiments with intravenous copper acetate and blocking agents. The results are summarized in Table 3. It is apparent that copper activation of the hypophysis occurs in spite of Dibenamine, atropine or Nembutal. The large number of Dibenamine-injection time-intervals was undertaken because of Brooks's (1940) report that copper-activa-

tion was blocked in some cases by stalk section up to 5 hours after injection. Special efforts were made to insure that the blocking activities of the drugs should not wear off before the copper effect was exerted: subcutaneous atropine was substituted for intravenous, and Nembutal anesthesia was maintained in 5 animals for more than 8 hours after copper injection. Two of the 3 rabbits which failed to ovulate in the Dibenamine and atropine series possessed relatively immature ovaries but they had responded 3 weeks earlier to copper alone, one with ruptured and the other with hemorrhagic follicles; the third was not tested.

TABLE 3. INEFFECTIVENESS OF BLOCKING AGENTS TO PREVENT COPPER-INDUCED OVULATION IN ESTROGEN-TREATED RABBITS. EACH RABBIT RECEIVED 10 MG. COPPER ACETATE INTRAVENOUSLY

Number of animals	Blocking Agent				Results		
	Type	Dosage mg./kg.	Route	Time of injection relative to copper	Activation of hypophysis (ovulation or hemorrhagic follicles)	Failed to activate	Percentage activated
12	—	—	—	—	12	0	100
7	Dibenamine	30	i.v.	1 hr. before	6	1	86
4	Dibenamine	30	i.v.	1 min. before	4	0	100
8	Dibenamine	30	i.v.	0.5, 1.5, 3, 6 hrs. after	8	0	100
6	Atropine	30	i.v.	1 min. before	5	1	83
6	Atropine	300	sub. cut.	1 hr. before	5	1	83
7	Nembutal	90–135	i.v. and sub. cut.	1 min. before and up to 8 hrs. after	7	0	100

The Nembutal experiments confirm the finding of Brooks *et al.* (1940) that an anesthetic dose of Nembutal fails to block copper-induced ovulation, though our results do not endorse the application of these findings to the picrotoxin problem as was done by Brooks *et al.* and Kasdon (1949). It was possible in the earlier work that copper exerted its effect after recovery from the relatively brief Nembutal anesthesia, but the likelihood of such an occurrence has been largely removed in the present study by the long-continued anesthesia.

The possibility also existed that Dibenamine and atropine failed because copper had in some way inactivated their anti-adrenergic and anti-cholinergic properties respectively. This contingency was tested in the case of Dibenamine with 4 rabbits by following Dibenamine and copper injections with twice-lethal intravenous doses of epinephrine (1 mg./kg.) the same day and for several days thereafter. All 4 of the animals were not only protected for several days from the lethal effects of epinephrine but they even failed to show any observable symptoms of epinephrine poisoning. Similarly, 4 rabbits injected with atropine subcutaneously and copper intravenously failed to show symptoms to twice-lethal intravenous doses of acetylcholine-eserine solution (450 μg.–225 μg./kg. respectively) given 3 to 5 hours after the atropine. It is safe to say that, in the presence of blocking agents, copper exerts its stimulatory effect on the hypophysis at a time when

Dibenamine, atropine, and Nembutal are still actively anti-adrenergic anti-cholinergic and anesthetic, respectively.

The blocking agents failed even to delay copper activation of the hypophysis: ovulation, induced by the metal in the presence of the drugs, had already occurred by 24 hours after injection in the few animals whose laparotomies were performed at that time. Nor did the blocking agents interfere with the formation of corpora lutea or the normal duration of pseudopregnancy.

We are forced by these data to choose between the two following assumptions: either (1) copper activates a neurogenic stimulation of

TABLE 4. ATTEMPTS TO INDUCE OVULATION IN ESTROGEN-TREATED RABBITS BY INJECTING COPPER ACETATE DIRECTLY INTO THE HYPOPHYSIS

Number of animals	Total dosage of cu-acetate mg.	Site of copper, as determined at autopsy	Results		
			Activation of hypophysis (ovulation or hemorrhagic follicles)	Failed to activate	Percentage activated
12	0.15	Extrahypophyseal	0	12	0
8	0.15	Intrahypophyseal	6	2	75

the hypophysis qualitatively different from that initiated by coitus, since the latter is the more potent stimulus (Emmens, 1940) yet is blocked by Dibenamine and atropine; or (2) copper exerts a stimulatory influence directly on cells of the adenohypophysis. The first assumption is difficult to test experimentally, but an investigation of the possible direct action of copper has been made and the results are presented below.

Experiments on the intrahypophyseal injection of copper acetate. The results are summarized briefly in Table 4. In 12 of the 20 experimental animals the copper could not have reached the hypophysis directly for the hypophyseal capsule was found at autopsy to have remained intact. None of these 12 gave ovarian evidence of pituitary activation. The group included 5 whose copper acetate solution contained India ink. At autopsy considerable ink was found in the retropharyngeal region below the sella in all 5 animals, and 2 also revealed ink in their cavernous sinuses.

Of the 8 animals whose hypophyses were penetrated, 6 ovulated. In 4 of the 6 the penetration was partial, and there seemed very little possibility that the copper could have reached the hypothalamus (Fig. 2). The other 2 had hypophyses perforated to the top in such a way that some of the copper could possibily have passed the diaphragma sellae (Fig. 3). Three of the 8 penetrated hypophyses, including 2 from animals that ovulated, were entered with the copper-ink medium, but in all 3 most of the ink was found inferior to the sellar region, none above the diaphragma sellae (Fig. 4).

Of the 2 penetrated hypophyses which were not activated, the

one receiving ink with its copper was penetrated only shallowly with the ink but revealed considerable hemorrhage (but not ink) about the median eminence (Fig. 5). The other was penetrated in the zona tuberalis and most of the adenohypophysis behind this region was necrotic, presumably due to interference with its blood supply (Fig. 6). The ovaries of neither animal gave any gross or histological evidence of pituitary stimulation. It seems entirely possible that, with vascular damage at the time of stimulation, the activated hypophysis might not be able to release its hormone in sufficient amounts to affect the ovarian follicles.

Three series of experiments, reported previously, serve as controls and demonstrate that mechanically and chemically induced trauma of the hypophyseal cells does not stimulate the release of luteinizing hormone. Seven rabbits' hypophyses received bipolar electrodes without ovulating (Markee, Sawyer and Hollinshead, 1946). The hypophyses of 23 females were injected with acetylcholine-eserine solution and 9 with potassium cyanide (Markee, Sawyer and Hollinshead, 1948). None of the latter and only one of the former rabbits ovulated.

DISCUSSION

Our results with picrotoxin confirm those of earlier investigators (Marshall *et al.*, 1939; Brooks, *et al.*, 1940; Kasdon, 1949), who considered that this stimulant induced ovulation in the estrous rabbit by excitation of the central nervous system. The ultimate, if not the primary, site of this nervous stimulation must be the hypothalamus, for several investigators (cf. Harris, 1948a) have shown that the hypophysis is activated by the hypothalamic application of electrical stimuli that are too weak to be effective on other parts of the nervous system or even on the adenohypophysis (Markee, Sawyer, and Hollinshead, 1946) or the infundibular stalk (Harris, 1948b). There is indirect evidence which indicates that the hypothalamus is the primary site of picrotoxin stimulation: Schriever and Perschmann (1935) found this region in the frog to be especially sensitive to the drug, as evidenced by its low threshold for convulsions. In the present study convulsions appeared to be a prerequisite to the induction of ovulation by picrotoxin as had been true in the work of Brooks, *et al.*, and Kasdon but not in the report of Marshall *et al.*

The present work confirms the finding of Marshall *et al.*, that the threshold for pictrotoxin-convulsions is lowered by preliminary atropinization and reveals that the threshold is similarly affected by Dibenamine. In the presence of blocking dosages of atropine or Dibenamine the convulsions incited by the reduced amounts of picrotoxin were actually of greater severity than those induced by the higher levels of picrotoxin alone. Indeed, it was necessary to employ 21 rabbits to obtain 10 survivals of convulsions in the atropine and Dibenamine series. The narrowness of the picrotoxin dose-range between merely convulsive and convulsive-lethal dosages in the "blocked"

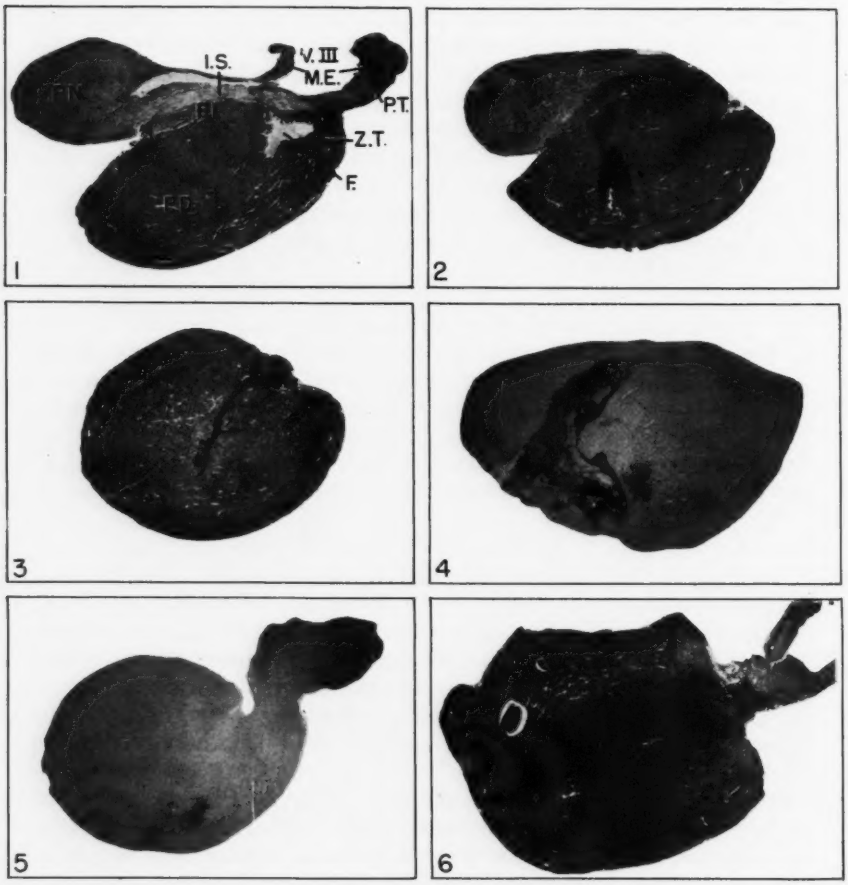

PLATE 1. EXPLANATION OF FIGURES

All of the sections are cut in approximately the same plane and all are oriented so that the median eminence is, or would be if present, in the upper right quadrant. Figures 1, 2, 3 and 6 have been stained with the hematoxylin tri-acid method and figures 4 and 5 by acid fuchsin only. The photographs have been cut out and remounted but are not retouched. Magnification, ×12.5.

FIG. 1. Mid-sagittal section of a control hypophysis. The hypophyseal capsule was not perforated and the rabbit failed to ovulate. In removal the pars intermedia partially broke away from the pars distalis. F.—fossa between zona tuberalis and remainder of pars distalis. I.S.—infundibular stalk. M.E.—median eminence. PD.—pars distalis. P.I.—pars intermedia. P.N.—pars nervosa. P.T.—pars tuberalis. V.III—third ventricle. Z.T.—zona tuberalis. The portal veins enter the pars distalis via the zona tuberalis.

FIG. 2. Section of an hypophysis which was activated by copper entering the pars distalis and reaching but not penetrating, the pars intermedia.

FIG. 3. Parasagittal section of an hypophysis completely pierced by the pipette, showing the superior point of exit. In this case and one similar to it, the copper may have reached the hypothalamus.

FIG. 4. Mid-sagittal section of an hypophysis penetrated with copper dissolved in

series, coupled with the fact that these dosages were at best low when compared with those in the "non-blocked" series, argued against further extension of the atropine and Dibenamine experiments. However, if the severity of convulsions is assumed to be a more important element in pituitary activation than the picrotoxin dosage level (see below), and if the ratios of pituitary activation following Dibenamine (0/5) and atropine (1/4) are grouped, the resulting ratio of 1/10 is significantly different from the 6/12 ratio of activation in the "non-blocked" series.

The Nembutal-picrotoxin series requires no such assumptions to establish its activation ratio (0/7) as significantly different from the 6/12 ratio in the picrotoxin control series ($P<0.01$); the picrotoxin dosage after Nembutal was more than twice as great as in the control series. The experiments demonstrate conclusively that Nembutal blocks picrotoxin-induced ovulation and suggest that the mechanism of blocking is related to the inhibition of convulsions. Quite possibly picrotoxin and Nembutal both act preferentially on the hypothalamus and are mutally antagonistic; picrotoxin is recognized as a valuable antidote in barbiturate poisoning (Tatum, 1939). The fact that Brooks *et al.* (1940) and we employed Nembutal secondarily to prevent picrotoxin-convulsions from killing the rabbits probably accounts for our relatively reduced percentages of ovulations compared with those of Marshall *et al.*, who did not use Nembutal.

The observation that Nembutal blocks picrotoxin-activation of the hypophysis assumes increased significance with the discovery (Everett and Sawyer, 1949) that in rats Nembutal blocks the natural neurogenic stimulus leading to an ovulatory discharge of LH from the adenohypophysis (Everett, Sawyer and Markee, 1949). We have so far failed to block copulation-induced ovulation in the rabbit with Nembutal (Sawyer, Markee and Hollinshead, 1947, and unpublished) most likely because the central or hypothalamic phase of the copulation-pituitary activation reflex is completed before the post-coitally injected Nembutal can become effective. Under similar conditions both atropine and Dibenamine block activation of the hypophysis after mating in the rabbit probably because they attack elements on or near the efferent end of the reflex arc (Sawyer, Markee and Townsend 1949). The fact that Dibenamine and atropine appear to block picrotoxin-activation of the hypophysis, while facilitating the convulsions, is further evidence that the site of blocking action of these drugs is efferent rather than central.

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an India ink suspension. The ink medium was found in the pars distalis and throughout the intermedia. The rabbit ovulated.

FIG. 5. Oblique section lacking posterior lobe and infundibular stalk but with zona tuberalis connecting median eminence and pars distalis. India ink penetrated the distalis shallowly but there was considerable hemorrhage about the median eminence. The animal failed to ovulate.

FIG. 6. Oblique section showing necrosis resulting from perforation of the zona tuberalis and destruction of the blood supply. The rabbit failed to ovulate.

Neither the dosage of Nembutal which blocked picrotoxin-induced ovulation nor dosages of Dibenamine or atropine that blocked pituitary activation by either mating or picrotoxin, had any significant effect on stimulation of the release of luteinizing hormone by copper acetate. The results of experiments with copper and these three blocking agents, ovulation in 35 out of 38 rabbits, compares favorably with the results of non-blocked mating-experiments, ovulation in 28 out of 30 mated females (Sawyer, Markee and Everett, 1950). From what we have seen of the action of Nembutal in blocking the natural neurogenic stimulus in the rat and the picrotoxin effect in the rabbit, it would seem probable that if copper were stimulating afferent or central nervous mechanisms its action would be blocked by Nembutal. If it were stimulating efferent neural elements, the copper effect should be blocked by atropine or Dibenamine. Since copper stimulation was blocked by none of these agents we were led to the conclusion that copper exerts it principal effect, not on the nervous system, but upon hypophyseal cells themselves. Our results do not deny that copper may exert other influences on the nervous system.

Our results of injecting 0.15 mg. copper acetate directly into the adenohypophysis may be compared with those of Harris (1941) who injected 0.05 mg. into the third ventricle. Six out of 8 of our rabbits ovulated whereas Harris reported positive results in 10 out of 13 cases. The minimal ovulating dose when injected intravenously is about 10 mg. copper acetate, so the dose used by Harris was of the order of 1/200, and that used by us, 1/70 of the systemic dose. Most of our larger dose, however, backed out of the sella away from the hypophysis and hypothalamus, and in only 2 of the 6 positive cases did there appear to be any possibility of copper reaching the hypothalamus except as it was absorbed into the systemic circulation. On the other hand, the copper injected by Harris into the third ventricle and assumed by him to have stimulated the hypothalamus, could as readily have stimulated the hypophysis directly. The proximal capillary plexus of the hypophyseal portal system becomes subependymal in the median eminence at the infundibular recess of the third ventricle (Harris, 1947), and the direction of blood flow in the portal system is unquestionably from the median eminence to the adenohypophysis (Green and Harris, 1949). Soluble substances in the third ventricle would have to pass only through ependyma and capillary endothelium to reach this vascular thoroughfare to the pars distalis; actually this process would require less diffusion than to reach most of the hypothalamic nuclei.

The success of Brooks *et al.* (1940) in blocking copper-induced ovulation by severing the hypophyseal stalk is difficult to explain in terms of a direct action of copper on pituitary cells, since cutting the stalk does not remove the arterial supply of the adenohypophysis (Brooks, 1938; Harris, 1947). However, since it is difficult to ascertain whether a female rabbit is in heat unless she is mated (Bradbury,

1944a) and since Brooks injected no estrogen, it is possible that his copper-treated stalk-sectioned animals were anestrous.

If copper exerts its primary influence directly on hypophyseal cells, where does estrogen facilitation occur? Dury and Bradbury (1943) suggested that "copper is an effective stimulus only when some mechanism (possibly neural) has been activated by estrogen." In the rat, neurogenic stimulation of the release of LH is facilitated by estrogen, and it was suggested that the estrogen effect is exerted at a gonadotrophic "sex center" in the hypothalamus (Sawyer, Everett and Markee, 1949). Recent work in the rabbit has shown that the reflexogenic induction of ovulation by mechanical stimulation of the vagina is also facilitated by estrogen (Sawyer, 1949). Pseudopregnant rabbits do not ordinarily ovulate in response to coitus, but by supplying estrogen Klein (1947) was able to stimulate ovulation by mating during pseudopregnancy. We have confirmed this and have found that copper is an even more effective stimulus to the estrogen-treated pseudopregnant animal than is mating; the results of these experiments will be reported in a separate communication.

A new hypothesis is necessary to harmonize the facts of neural and copper stimulation: that estrogen lowers the thresholds to both neurogenic and copper stimulation at the hypophysis itself. Since neural stimulation involves a chemical mediator, the two types of stimuli are not fundamentally different. The natural final mediator appears to be an adrenergic one (Sawyer, Markee and Townsend, 1949). The new hypothesis does not deny that estrogen exerts profound influences on the central nervous system but merely suggests that, as far as the control of the hypophysis is concerned, the steroid exerts an important conditioning influence on the gland itself.

SUMMARY AND CONCLUSIONS

A study of the mechanisms by which picrotoxin and copper salts induce ovulation in the rabbit introduces evidence that, whereas picrotoxin definitely functions by way of the nervous system, at least a large part of the copper stimulus is exerted directly on hypophyseal cells.

Picrotoxin-induced ovulation was always preceded by convulsions; in their absence pituitary activation failed to occur. Reduced dosages of picrotoxin led to extremely severe convulsions in rabbits previously treated with Dibenamine or atropine, but ovulation followed in only one case in 10 animals so treated. These results are interpreted as evidence that the nervous stimulus was generally blocked from reaching the hypophysis. Nembutal in anesthetic doses blocked the convulsive and pituitary-stimulating actions of doses of picrotoxin 2 to 4 times the minimal stimulating level.

Ovulation was induced by copper acetate in dosage which produced no overt signs of nervous stimulation. The copper stimulus to ovulation was blocked by neither the anti-adrenergic, the anti-cholin-

ergic nor the anti-convulsive agent. Finally, pituitary activation was achieved by injecting tiny amounts of copper acetate directly into the adenohypophysis.

Preliminary treatment with estrogen facilitates both neurogenically and copper-induced ovulation. It is suggested that the steroid acts at the hypophyseal level by lowering thresholds to both neurohumoral and direct chemical stimulation.

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PARTIAL MAINTENANCE OF THE ADRENAL CORTEX BY ANTERIOR PITUITARY GRAFTS IN FED AND STARVED GUINEA PIGS

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CONSIDERABLE interest has centered about the question of the nature of the stimulus to the anterior pituitary gland which leads to the release of adrenocorticotrophic hormone (Long, 1947; Sayers and Sayers, 1948). There are two phases to this problem; first, the stimulus which results in the release of adrenocorticotrophin in the normal animal and maintains adrenal cortical function at customary levels; and second, the stimulus which mobilizes the additional trophic hormone needed rapidly during conditions of stress in which there is increased utilization of cortical hormone. The mechanisms involved are not necessarily the same. Uotila (1939) and Dempsey and Searles (1943) have shown that whereas section of the hypophyseal stalk in the rat does not interfere with maintenance of the thyroid, the release of extra thyrotrophin in response to cold which occurs in the intact rat is absent in the stalk-sectioned animal. However, the adrenal which is also maintained in the stalk-sectioned rat responds to the stress of cold with the release of additional adrenocorticotrophin. The adrenal cortical response to acute stress (histamine) is also similar in the intact and stalk-sectioned rat (Cheng *et al.*, 1949). Recent evidence (Long, 1947) indicates that the stimulus to the hypophysis for the mobilization of extra adrenocorticotrophic hormone during stress is mediated by the adrenal medulla. Sayers and Sayers (1947) on the other hand, relegate epinephrine to the position of a form of stress which reduces the level of cortical hormone in the blood. They believe the direct stimulus to the pituitary is the level of circulating cortical hormone and have shown that prior treatment with cortical hormone prevents the fall in ascorbic acid after cold, painful stimuli and epinephrine. In earlier studies on the maintenance of adrenocortical tissue by intraocular grafts of anterior pituitary tissue we obtained conflicting results (Schweizer, Charipper and Haterius, 1937; Schweizer, Charipper and Kleinberg, 1940). It seemed of interest to re-examine this problem using certain indicated changes in technique.

A possible cause of the failure to maintain adrenal tissue in some

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of these experiments (Schweizer *et al.*, 1940) was the fact that implantation and hypophysectomy were performed at the same time. During the first few days there is evidence of partial regression of the graft and a gradual growth of vessels into the implant. While it is becoming established the trophic hormones are probably available only in very small amounts. An ovary removed three weeks after operation from an hypophysectomized guinea pig with a pituitary implant showed degenerative changes whereas the remaining ovary removed several weeks later was stimulated to overproduction of estrogenic hormone (Schweizer *et al.*, 1937). It seemed likely that similar regressive changes in the adrenal during the initial stages of the experiment may have been more difficult to reverse and that insufficient adrenocorticotrophin was secreted by the implant to bring about regeneration of the atrophied tissue. In incompletely hypophysectomized guinea pigs bearing implants, the total amount of pituitary tissue was sufficient to maintain the normal weight of the testes but the adrenal atrophy was as great as in the totally hypophysectomized controls (Schweizer *et al.*, 1940). It is known (Evans, Pencharz, Meyer and Simpson, 1933) that larger quantities of hormone are required to cause regeneration of the atrophied adrenal in the hypophysectomized rat than to maintain the adrenal when injections are begun immediately after operation. Large amounts of a potent adrenocorticotrophic principle (Ingle, 1942) failed to stimulate regeneration of the enucleated adrenal in the hypophysectomized rat. Six times as much adrenocorticotrophin was needed to restore the adrenal four weeks after hypophysectomy as was necessary after one week (Reiss, 1947).

In the present series of experiments an attempt was made to meet this difficulty by postponing hypophysectomy until the transplant seemed well established. Various intervals between implantation and hypophysectomy were tried; the most favorable period seems to be a week. The following experiments based upon the criteria of organ weights and alterations in sudanophilic material indicate that such grafts are capable of giving some support to the adrenal cortex though they do not maintain it at normal levels.

EXPERIMENTAL PROCEDURE

Adult male and female guinea pigs (400–600 grams) were used in these experiments. The anterior pituitary gland of the donor was bisected and one half of the gland implanted in the anterior chamber of each eye of a guinea pig of the same sex as the donor. The implants were examined daily until the graft seemed well established; the pituitary of the host was then removed by the parapharyngeal approach. The interval between implantation and hypposectomy varied from one to four weeks. The animals were kept at the usual laboratory temperature (70–80° F) and were given purina chow supplemented

daily by ample amounts of greens; water was available at all times.

Blood for the white cell counts was obtained by puncturing an ear vein with a surgical needle. Total white cell counts were done by the usual technique, counting the cells on both sides of a Neubauer chamber. The differential counts were based on a study of 500 cells on dried smears using Wright's stain.

Animals were sacrificed by exsanguination after a brief exposure to ether. Organs were carefully trimmed, weighed to the nearest milligram on a torsion balance and placed in suitable fixatives. The contents of the sella were examined under a binocular microscope and all fragments were fixed and sectioned. The implants were removed by dissection under a binocular microscope and fixed with the small portion of the iris to which they were generally adherent.

The left adrenals, used for this report, were fixed in neutralized 10% formalin. After fixation for two or three days they were embedded in gelatin (Zwemer, 1933), sectioned on the freezing microtome at 10 and 15 μ, stained with sudan black B (Baker, 1944) and mounted in glychrogel. Representative sections from the mid region of the gland were examined microscopically to determine variation in the distribution, size and number of sudanophilic lipid droplets in the zones of the cortex. Whitehead (1934) demonstrated that there was no statistical difference in sudanophilic area between 20 such representative sections taken from either gland.

Measurements of variations in the width of the glomerulosa and of the distribution of sudanophilic droplets in the cortex were made with an ocular micrometer. The mean glomerulosal width for each adrenal was obtained by calculating the mean of ten measurements around a section. The measurements, converted to micra are presented in Table 1. The width of the sudanophilic zone and of the cortex was determined by ocular micrometer measurements of from five to ten regions (depending upon the uniformity of lipid distribution) on one radius of each section. The portion occupied by sudanophilic droplets was expressed as a per cent of the cortical width for each site measured, and the mean calculated for each gland. Glomerulosal lipid droplets were not included in the sudanophilic measurements because in the guinea pig their total lipid content is small (Whitehead, 1934).

In choosing the sites for cortical width measurement, the shape of the guinea pig adrenal was taken into consideration. The left adrenal is roughly triangular in section. At the apices of the triangle the fasciculata and reticularis are usually irregular in their relative distribution. Therefore, for measurement, only regions on the sides of the triangular-shaped sections were utilized.

The sella fragments, the implants, and normal pituitaries for comparison were fixed in formol-Zenker, sectioned serially at 5 μ and stained by a modification of Masson's Poinceau-Acid Fuchsin and Anilin Blue differential stain.

TABLE 1. CONTROL GROUPS

Experimental procedure	No. of animals F = female M = male	Body wgt. gm.	Rel. adr. wgt mg./100 gm. ± average deviation	Glomerulosa width μ (range)	Fasciculata sudanoph. zone % cortex width (range)	Glomerulosa droplets
Normal	10 6F 4M	466	50.5± 7.3	50 (40–60)	75 (60–80)	numerous fine
Hypx. 2 wks.	3 2F 1M	557[1]	33.9± 5.2	60 (55–80)	50 (35–55)	few fine
Hypx. 3 wks.	3 1F 2M	567[1]	39.4±13.1	70 (65–80)	45 (40–55)	few fine
Hypx. 4 wks.	3 1F 2M	546[1]	29.3± 2.9	75	35 (20–40)	few fine
Hypx. 6 wks.	3 1F 2M	506[1]	30.9± 9.8	80 (65–80)	30 (20–35)	few very fine
Implanted Pit. 1 wk.	4 2F 2M	541	68.8±17.4	—	Lipid changes varied	
Implanted Pit. 3 wks.	1 F	600	56.3	50	55	numerous fine
Implanted Muscle 1 wk.	6 4F 2M	451	51.4± 9.2	—	—	—
Normal starved wgt. loss 35 p.c.	2 M	500[2]	63.4± 9.2	25	55 (40–60)	very numerous fine & medium
Hypx. 3 wk. Starved wgt. loss 35 p.c.	3 M	525[1]	29.6± 5.5	55 (40–65)	50 (45–55)	very numerous fine & medium
Partially Maintained						
Implanted 6 wks. Hypx. 4 wks.	62[3] F	525[1]	43.4	70	60	numerous fine
Implanted 6 wks. Hypx. 4 wks.	21[3] M	490[1]	34.8	70	50	numerous fine
Implanted 7 wks. Hypx. 6 wks.	12[3] F	620[1]	85.6	80	65	numerous fine
Implanted 5 wks. Hypx. 3 wks.	67[3] F	525[1]	33.3	80	70	less than normal fine
Implanted 4 wks. Hypx. 3 wks. Starved wgt. loss 39 p.c.	49[3] F	520[1]	39.4	50	55	numerous fine & medium
Implanted 4 wks. Hypx. 3 wks. Starved wgt. loss 34 p.c.	50[3] F	480[1]	54.6	50	75	numerous fine & medium
Not Maintained						
Implanted 5 wks. Hypx. 3 wks.	66[3] F	550[1]	28.4	80	40	few fine
Implanted 5 wks. Hypx. 4 wks.	65[3] F	470[1]	48.1	65	30	few fine
Implanted 6 wks. Hypx. 4½ wks.	63[3] F	550[1]	36.7	80	30	very few fine
Implanted 12 wks. Hypx. 8½ wks.	5[3] F	410[1]	43.9	70	30	very few fine
Implanted 13 wks. Hypx. 10 wks.	3[3] F	655[1]	33.9	65	30	none visible
Implanted 14 wks. Hypx. 10½ wks.	1[3] F	580[1]	29.6	70	40	none visible

[1] Weight at hypophysectomy.
[2] Weight before starvation.
[3] Animal number.

Thirty-five guinea pigs (12 males, 23 females) were implanted with the pituitary of a donor of the same sex. Four of these developed eye infections and are not included in this report. Four (2 males, 2 females) were sacrificed after one week to see the effect of the extra pituitary on the adrenal gland. One was sacrificed three weeks after implantation to see whether the stimulating effects found in the first week had subsided. The remaining twenty-six were hypophysectomized at various intervals (indicated in Table 1) after implantation. Twelve of these (approximately 50 per cent) died within one week after hypophysectomy. The others were sacrificed at intervals from three to ten and one half weeks after operation. The only two cases which had small fragments of anterior lobe tissue in the sella at autopsy are not included in the table.

Ten animals (3 males, 7 females) were implanted with fragments of skeletal muscle comparable in size to the pituitary gland. These were used to distinguish the effect of the stress of the implantation procedure *per se* from the stimulating effect of the pituitary during the first week. Two which developed eye infections are not included in the report. Six were sacrificed one week after implantation (see Table 1). Two which were sacrificed three weeks after implantation are not included in the table as they did not differ from those sacrificed at one week.

Twenty-five (14 males, 11 females) guinea pigs were hypophysectomized to serve as controls. Ten died during the first week after operation. The remaining fifteen were sacrificed in groups of three at intervals from two to six weeks after operation (Table 1).

The effects of starvation were studied by placing the animals in individual metabolism cages and withholding all food; water was available at all times. Two normal, three hypophysectomized and two implanted hypophysectomized animals were starved until they had lost about 35 per cent of their body weight (approximately 7 days). At the end of the starvation period the experimental animals had been hypophysectomized three weeks.

RESULTS

Relative Weight of the Adrenal Gland:

Mixner, Bergman and Turner (1943) report no significant difference in the adrenal weight between the male and female guinea pig. Our observations are in agreement with this. In a group of 25 animals we found that 10 males (mean body weight 461 gm.) had a relative adrenal weight of 51.4 ± 2.8;[1] for 15 females (mean body weight 462 gm.) the relative adrenal weight was 54.9 ± 4.0. The mean of the ten normal animals (4 males, 6 females) reported in Table 1 does not differ significantly from these.

[1] Weight in mg./100 gm. ±Standard Error.

In reporting the relative adrenal weight of the experimental animals the adrenal was related to the body weight at the time of operation in those animals which were hypophysectomized, and to the weight at the beginning of the experiment in those normal animals which were starved. This method of calculation eliminated spurious effects resulting from the marked variation in the weight changes of different hypophysectomized animals. Some lost weight (especially during the first week), some maintained a constant weight and a few showed a significant increase. It seemed more consistent to relate the adrenal weight to the body weight of the animal when its adrenal was still under pituitary control. Similarly in the starved animal the extreme weight loss gave a false impression of hypertrophy since the adrenal measured was actually that of a much larger animal.

The relative adrenal weight dropped about thirty per cent by the second week of hypophysectomy and changed only slightly after that. At later stages of hypophysectomy there was little change in the lower limits but the range narrowed. In this experiment the average deviation was particularly large in the group sacrificed three weeks after hypophysectomy. Such variation can occur as a result of chance selection of animals at one extreme or another of the normal range. The decrease in relative adrenal weight in the guinea pig is not as rapid as in the rat. Deane and Greep (1946) found a 30 per cent drop in relative adrenal weight in about one week and a 50 per cent drop by the second week in the hypophysectomized rat.

The addition of the pituitary implant in the normal animal increased the relative adrenal weight about 35 per cent during the first week. However, since the standard error of the difference of the mean of this group from the normal is 11.0 ($p = .099$) the increase is of questionable significance statistically. That the increase is due to the effect of the pituitary and not merely to the stress of the implantation procedure is indicated by the fact that implants of muscle present for the same interval caused no alteration of adrenal weight.

The relative adrenal weight as a criterion for maintenance is indicative but not conclusive. Adrenal weights at the low end of the normal range are difficult to evaluate on this basis alone. Some of the adrenal weights in the group which were considered partially maintained on the basis of lipid distribution do not differ significantly from some in the group considered not maintained. Only one animal (No.12) in the group which was partially maintained had an adrenal which was significantly heavier than normal.

Starvation caused an increase in the relative adrenal weight of the normal but not in the hypophysectomized animal. Since the adrenal weights of implanted, hypophysectomized animals are so variable it it is not possible to tell whether starvation modified their weights in any way.

The White Blood Cells:

In the light of the reports of the influence of adrenocorticotrophin on the circulating lymphocyte (Dougherty and White, 1940; White and Dougherty, 1945) it was hoped that the total lymphocyte count might give an indication of the extent of adrenal cortical activity. No attempt was made to distinguish between monocytes and lymphocytes because of the extreme variability of these forms in the guinea pig. The results are summarized in Table 2.

TABLE 2. WHITE BLOOD CELL COUNTS

Experimental procedure	No. of animals	Total white blood cells ±standard error	Total mononuclear cells ±standard error	Percent mononuclear cells ±standard error
Normal	15	11,730± 380	7,380±440	62.6±1.9
One hour after hypophysectomy	10	15,500±1,577[1]	7,170±611	49.6±4.4[2]
Hypophysectomized one week	10	11,219± 781	7,585±582	67.2±2.8
Hypophysectomized three weeks	10	13,100±1,256	8,950±625	69.4±2.5
Implanted one week	8	12,887±1,138	7,644±374	61.8±4.1

[1] Significant difference from normal $p = .02$
[2] Highly significant difference from normal $p < .01$

In view of the well known lability of the white cell picture, the consistency of the values was surprising. The only significant change occurred as a direct result of operative procedures. One hour after hypophysectomy the total white cell count had increased 30 per cent but this was due entirely to an increase in polymorphonuclear cells as the monoclear count remained constant. All values had returned to normal by one week after hypophysectomy. The slight increases shown three weeks after hypophysectomy are not statistically significant. Even the implantation of the pituitary which in the normal animal results in an increase of adrenal weight at the end of one week did not reflect its effect by a change in the white blood cell count. The effect described by Dougherty and White occurred in acute experiments and may not necessarily be expected in the case of chronic stimulation of the adrenal.

Counts made on animals which were both implanted and hypophysectomized for various times are not reported as they presented an extremely inconsistent picture. In most instances the counts were similar for long periods but large fluctuations sometimes occurred and these could not be related in any way to the experimental situation at the time.

Distribution of Lipid

Sudan black B has been used to visualize the lipid droplet constitution of the cortical cells. Other sudan stains (sudan III, sudan

IV and their modifications) were found to give a generally paler coloration and appeared to demonstrate less sudanophilic material especially in the inner cortical regions (Harrison and Cain, 1947). Preliminary studies of the cholesterol distribution in the adrenal cortex of the guinea pig using the Schultz modification of the Liebermann-Burchard reaction indicate that changes in the sudanophilic distribution and in the cholesterol distribution are parallel in the experimental animals.[2] Such alterations in the sudanophilic substance are considered most likely to be due to changes in the cholesterol ester material (Sayers and Sayers, 1948).

The adrenals of all normal control animals in the present experiment demonstrated a sudanophilic distribution throughout the fasciculate zone and in several cases the lipid distribution extended into the reticularis (Fig. 1). Hoerr (1936) states that the zona reticularis of the normal guinea pig is never free of lipid. The regions of cortical sudanophilic material measured included only those showing a continuous distribution without regard to size and number of droplets. Scattered, isolated, lipid-containing cells in the reticularis were disregarded.

The mean relative width of the sudanophilic zone in the control groups and in the individual experimental animals is summarized in Table 1. In the normal animal the lipid zone occupies from 60 to 80 per cent of the cortical width. This decreases gradually to a value of about 35 per cent by the fourth week after hypophysectomy. It is

[2] Details of these lipid studies will be published elsewhere.

All photomicrographs on this and the succeeding plate are of adrenal glands of adult guinea pigs, fixed in 10 percent neutralized formalin, embedded in gelatin, sectioned on the freezing microtome at 10 μ, stained with sudan black B and mounted in glychrogel.

FIGS. 1–5. Adrenal cortex. Medulla present in lower portions of each figure. ×35.

FIG. 1. Normal cortex. Sudanophilic lipid is evenly distributed throughout the fasciculata and extends into the reticularis. The region figured shows a sudanophilic zone 85 per cent of the cortical width, representing a lipid fasciculate zone in its widest normal cortical distribution. The glomerulosa, width 50 μ, contains numerous fine sudanophilic droplets.

FIG. 2. Cortex of guinea pig No. 62, implanted 6 weeks, hypophysectomized 4 weeks, partially maintained. The irregular sudanophilic zone is 60 per cent of the cortical width. Compare with the hypophysectomized control, Fig. 3. The glomerulosa, width 70 μ, is similar to that of the hypophysectomized control but approximates the normal in lipid droplet distribution.

FIG. 3. Cortex of guinea pig hypophysectomized 4 weeks. The fasciculata has a sudanophilic zone 25 per cent of the cortical width (mean for the group, 35 per cent). The glomerulosa, width 75 μ, contains few, fine droplets, similar to Fig. 13.

FIG. 4. Cortex of guinea pig No. 12, implanted 7 weeks, hypophysectomized 6 weeks, partially maintained. The sudanophilic zone is 65 per cent of the cortical width. Compare with the hypophysectomized control, Fig. 5. The glomerulosa, width 80 μ, is similar to that of the hypophysectomized control but approximates the normal in lipid droplet distribution.

FIG. 5. Cortex of guinea pig hypophysectomized 6 weeks. The fasciculata has a sudanophilic zone 30 per cent of the cortical width. Glomerulosa similar to Fig. 3.

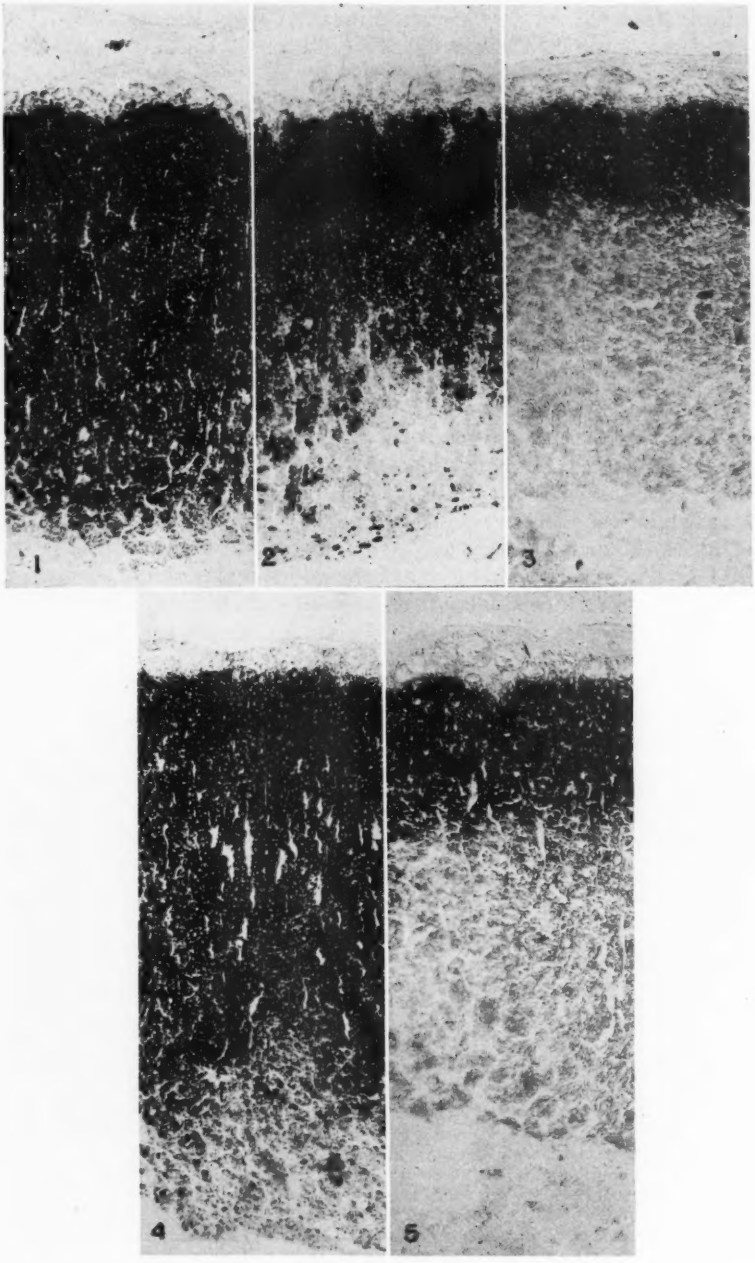

PLATE I

doubtful that this value differs significantly from the stable value of 30 per cent obtained at six weeks. It is of interest that after prolonged hypophysectomy in the guinea pig the sudanophilic zone occurs in the fasciculata whereas in the rat it occupies the glomerulosa (Deane and Greep, 1946).

Implanted hypophysectomized animals which were partially maintained have sudanophilic zones 30 per cent (No. 21) to 50 per cent (Nos. 62, 12, 67) wider than their hypophysectomized controls (Figs. 2, 3, 4 and 5). Those which were not maintained (Nos. 66, 65, 63, 5, 3, 1) are comparable with three to six week hypophysectomized controls (Figs. 9, 5 and 3).

Normal animals which were starved (Fig. 6) showed a decrease (about 25 per cent) in the width of the sudanophilic zone. There was also a marked reduction in the size of the individual fasciculata droplets, giving a less intense and more irregular distribution. None of these changes was apparent in the hypophysectomized starved animals (Fig. 8). Of the two implanted, hypophysectomized animals which were starved (Nos. 49 and 50), only one (No. 50) showed a sudanophilic zone which was significantly wider than the three week hypophysectomized and hypophysectomized starved controls (Figs. 7 and 8). There is no evidence that the additional stress of starvation has modified the adrenal glands of Nos. 49 and 50. They are partially maintained as are Nos. 62, 12, 21, 67, but on the basis of sudanophilic distribution in the fasciculate zone it is not possible to detect any effects of the starvation period.

FIGS. 6–9. Adrenal cortex; medulla present in lower portion of each figure ×35 Figs. 6, 7 and 8 are from starved animals. Fig. 9 from hypophysectomized control. Figs. 10–13, glomerulosa and outermost fasciculata rim of Figs. 6–9, respectively ×225.

FIG. 6. Cortex of normal starved guinea pig, weight loss 35 per cent. Irregular fasciculate sudanophilic zone, less intense than normal, is 55 per cent of the cortical width. The glomerulosa width 25 μ, narrower than normal (Fig. 1) and contains more abundant and larger lipid droplets (Fig. 10).

FIG. 7. Cortex of guinea pig No. 50, implanted 4 weeks, hypophysectomized 3 weeks, starved to weight loss 34 per cent, partially maintained. The irregular sudanophilic zone, less intense in inner fasciculata, is 75 per cent of the cortical width. (Compare with hypophysectomized controls, Figs. 8 and 9). The glomerulosa (Fig. 11) is broader (50 μ) than in the normal starved guinea pig (Fig. 10) but the lipid droplet distribution is similar.

FIG. 8. Cortex of guinea pig hypophysectomized 3 weeks, starved to weight loss 35 per cent. The fasciculata has an irregular sudanophilic zone, slightly less intense in inner fasciculata, 50 percent of the cortical width. The glomerulosal width (55 μ) and lipid distribution (Fig. 12) is similar to that in Fig. 11.

FIG. 9. Cortex of guinea pig hypophysectomized 3 weeks. The fasciculata has a sudanophilic zone 45 percent of the cortical width. The glomerulosa (70 μ) shows the widening and the depleted lipid that follows hypophysectomy (Fig. 13).

FIG. 10. Glomerulosa of normal starved guinea pig (Fig. 6).

FIG. 11. Glomerulosa of starved guinea pig No. 50 (Fig. 7).

FIG. 12. Glomerulosa of starved guinea pig, hypophysectomized 3 weeks (Fig. 8).

FIG. 13. Glomerulosa of guinea pig hypophysectomized 3 weeks (Fig. 9). Lipid depletion due to hypophysectomy is in striking contrast with increased lipid deposition of starvation (Figs. 10, 11 and 12).

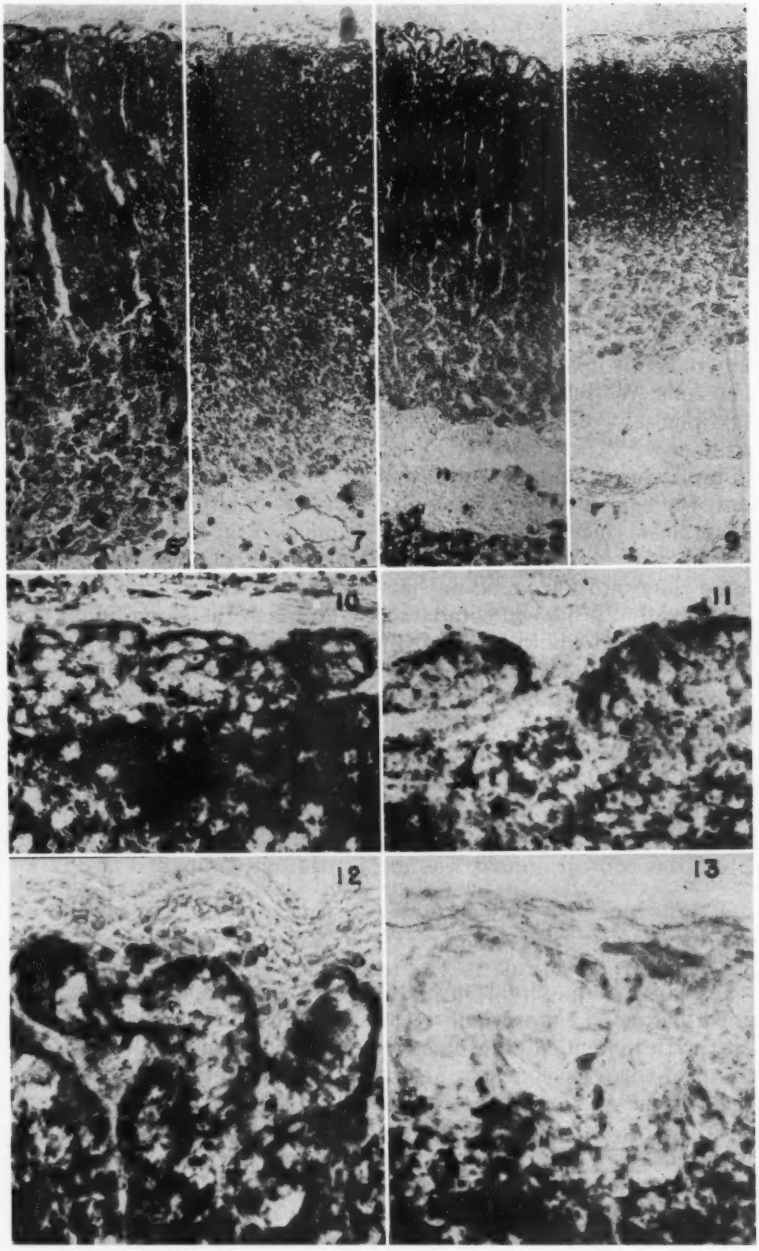

PLATE II

The Glomerulosa

The variations in glomerulosal width and the size and distribution of the lipid droplets is summarized in Table 1. The width of the glomerulosa increased gradually, reaching a constant condition about 50 per cent greater than normal by the fourth week after hypophysectomy. This increase in width of the zone is a very stable factor. It is found to have virtually the same value four weeks after hypophysectomy whether the animals were hypophysectomized controls, implanted hypophysectomized animals which were partially maintained or those which were not maintained. The only experimental condition which produced a significant change was inanition.

The glomerulosal cells of the normal animal (Fig. 1) contained numerous fine lipid droplets though occasionally these were not found in all cells. Whitehead, (1934), reported minute glomerulosal droplets in 83 per cent of his cases. The droplets become progressively fewer and finer with the time after hypophysectomy. In animals, nos. 1 and 3 (implanted, hypophysectomized 10 weeks, but not maintained) there were no droplets visible except occasionally in the innermost glomerulosal cells. The sudanophilic droplets of the implanted hypophysectomized animals which were partially maintained approximated the condition found in the normal animal.

Starvation produced comparable changes in the glomerulosa of all animals studied whether the pituitary was present or not. There was a decrease in glomerulosal width and a marked increase in the number and size of the sudanophilic droplets. The glomerulosal width of the normal animal decreased 50 per cent (Figs. 1, 6 and 10); that of the hypophysectomized (Figs. 7, 8, 11 and 12) and the implanted hypophysectomized animals (Figs. 7 and 11) narrowed about 20 per cent. The glomerulosal lipid after starvation seemed similar in the three groups though the change from the depleted state of the hypophysectomized control animal was most striking (compare Figs. 12 and 13).

Implants

Implants from animals on normal diet which were partially maintained, had good connections with the iris, exhibited extensive vascularity with distended sinusoids frequently engorged with blood cells and all of these except Nos. 21 and 67 showed the cell nest arrangement characteristic of anterior lobe tissue. Scar tissue was present in the central region of the grafts except in Nos. 62 and 12. The best maintained anterior lobe tissue was found in the peripheral portions of the implant in the region of fusion with the iris. Portions in contact with the cornea usually underwent degenerative changes. In the implant cells of Nos. 62 and 12, animals which had particularly well maintained adrenal tissue, all cell types were represented but

acidophils were much more numerous than the basophils. The basophils were paler in their staining reaction and were diagnosed as being in a degranulating or degranulated condition. The implants of Nos. 21 and 67 had decidedly fewer acidophils than Nos. 62 and 12, and were more chromophobic.

In the group which was not maintained, the implant tissue was disorganized in all but Nos. 1 and 66; the characteristic cell nests were evident in these two cases. The acidophilia was greatly reduced in all except Nos. 1 and 5. The implant of No. 1 had numerous acidophils and showed pale basophils and some chromophobes.

Implants from the partially maintained animals which were deprived of food, Nos. 49 and 50, were predominantly chromophobic. There were scattered small acidophils containing fewer and smaller granules than normal and some pale staining basophils.

One of the implanted hypophysectomized (three weeks) animals which was starved seven days (not reported in the table) had a fragment of anterior lobe tissue in the sella. A comparison of this fragment and the implant are of interest. The sella fragment of this animal (No. 51) showed the characteristic increase in chromophobic cells and basophils described by D'Angelo, Gordon and Charipper (1948). The implant, however, was predominantly acidophilic, though it also showed the presence of pale staining basophils and some chromophobes.

The sella fragment of an implanted hypophysectomized animal sacrificed $3\frac{1}{2}$ weeks after operation was stained concurrently with the implant of the same animal. The cell types of the anterior lobe tissue of the sella fragment were normal in relative numbers and in chromophobic granulation (Kirkman, 1937). All cell types were present in the implant but it had paler staining basophils.

The ability of an implant of anterior pituitary tissue to maintain the adrenal gland must depend upon both the qualitative condition of the gland and the total amount of tissue. The best maintained adrenals were in Nos. 12 and 62 which had well established implants of moderate size. Although the implants of Nos. 49 and 59 show some of the characteristic changes of starvation, No. 51 which had been starved the same length of time did not. It is difficult to understand this difference in response.

DISCUSSION

The increase in width of the glomerulosa in the guinea pig after hypophysectomy is in agreement with reports for the rat (Crooke and Gilmour, 1928; Deane and Greep, 1946; Deane, Shaw and Greep, 1948), but unlike the rat (Greep and Deane, 1947) the glomerulosa of the guinea pig shows a marked depletion of sudanophilic material. This condition of hypertrophy is found even in those animals otherwise showing evidences of partial maintenance by the pituitary grafts. The atrophy of this zone in the starved guinea pig occurs not

only in the normal animals (D'Angelo *et al.*, 1948) but in the hypophysectomized and implanted, hypophysectomized animal as well, though to a lesser extent. The atrophy is accompanied by a marked increase in both number and size of the sudanophilic droplets. This reaction of the glomerulosa may be due to the direct effect of inanition as Swann (1940), and Deane and Greep (1946) have suggested that this zone is autonomous and not under the influence of the anterior pituitary. The loss of the sudanophilic droplets in the hypophysectomized guinea pig in contrast with their persistence in the rat, and the persistence of these droplets in hypophysectomized animals showing partial adrenal maintenance due to the presence of the pituitary implant raises the question whether the autonomy is as complete in the guinea pig as it seems to be in the rat.

The question of which cell type in the anterior pituitary secretes adrenocorticotrophin is unsettled. Considerable evidence supports the view that it is the basophil (Severinghaus, 1936; Zeckwer, 1936; D'Angelo, Gordon and Charipper, 1948). Recently Finerty and Briseno-Castrejon (1949) have presented data indicating that it is the acidophil. Reese, Koneff and Akimoto (1939) found changes in both types of cells after adrenalectomy.

The distribution of cell types in the implants described above is difficult to interpret definitely in the light of one of these alternatives rather than another until the condition of the other endocrine organs in these animals has been studied. Furthermore, intraocular grafts of anterior pituitary tissue are extremely variable. During the first week they always undergo some reduction in size. After vascularity had been established they sometimes remain constant in area for several weeks but they may suddenly show signs of regression. Frequently, the implants which are sectioned will show necrosis in the central regions. In no case has the graft maintained the adrenal at normal levels. This could be due to the fact that divorced from its normal neural and vascular connections it is incapable of responding normally to the stimuli which call forth its secretions. It is equally likely that the difficulty of obtaining sizeable amounts of normal grafted tissue may account for the failure to achieve normal levels of activity.

The inanition experiments reported above leave unsettled the question of whether the implants are able to mobilize additional hormone under conditions of prolonged stress. The relative adrenal weights of Nos. 49 and 50 are within the normal range in one case and at the low end of the normal range in the other; whether they would have been lower if the stress had not been applied cannot be determined. The observed effects of starvation on the glomerulosa also occurred in the hypophysectomized animals and cannot be used as a test of pituitary function. The fasciculate zone shows partial maintenance but gives no clear evidence of additional stimulation because of the application of the stress.

Half of the implanted, hypophysectomized animals showed some degree of adrenal maintenance. This was reflected chiefly in the distribution of sudanophilic material in the glomerulosal and the fasciculate zone. One difficulty inherent in the experiment is that the criteria used did not produce sufficiently striking effects as early as two weeks after hypophysectomy to enable one to distinguish a partially maintained from an hypophysectomized animal at this early period. There is reason to believe that the implants were functioning at higher levels at this time. Experiments using criteria which can be applied at earlier intervals after implantation are in progress.

CONCLUSIONS

The grafts of anterior pituitary tissue maintain the sudanophilic droplets of the glomerulosa and the sudanophilic zone of the fasciculata at levels distinctly above the hypophysectomized controls but below that of the normal controls. The relative adrenal weight when at the low end of the normal range is not in itself an adequate criterion for maintenance. The increase in width of the glomerulosa which occurs after hypophysectomy is not affected by the presence of the implant. Whereas implanted hypophysectomized animals which were starved showed partial maintenance of the adrenal there was no evidence of extra stimulation.

SUMMARY

The effects of intraocular grafts of anterior pituitary tissue on the adrenal gland were studied in the hypophysectomized guinea pig.

The white blood cell count was remarkably stable under most circumstances and could not be used as a criterion for adrenal cortical function in these chronic experiments.

The relative weight of the adrenal gland decreased sharply after hypophysectomy. This change is less in the group of animals which were implanted but on the basis of this criterion alone it was not possible to distinguish those animals in which the adrenal was partially maintained from those in which there was no maintenance when the relative adrenal weight was at the low end of the normal range.

The glomerulosa widens after hypophysectomy. The presence of an implant had no influence on the glomerulosa width, even when it gave partial maintenance of the fasciculata and when the relative adrenal weight was normal or above normal.

The sudanophilic material of the glomerulosa is depleted and gradually disappears after hypophysectomy. This change did not occur in hypophysectomized animals which had implants capable of partially maintaining the adrenal cortex (judged by lipid distribution in the fasciculate zone), but similarly prepared animals which do not show adrenal maintenance lose their lipid droplets.

The sudanophilic zone of the fasciculata narrows to a stable width

about 35 per cent of the cortex by four weeks after hypophysectomy. Animals with implants giving partial maintenance have a lipid laden zone 30 to 50 per cent wider than their hypophysectomized controls.

Starvation to loss of 35 per cent of body weight resulted in a decrease in width and an increase in sudanophilic material in the zona glomerulosa. This occurred in all groups tested though the decrease in width was greater in the normal than in the hypophysectomized or the implanted, hypophysectomized aniamsls.

Implanted hypophysectomized animals which were starved showed a partial maintenance of the adrenal as judged by relative adrenal weight, and width of the sudanophilic zone, but there was not clear evidence of extra stimulation as a result of the application of the stress of inanition.

The implants were variable in their cell types. All types were present but in every instance the basophils were paler than those of the normal pituitary. The relative proportion of acidophils varied from being the most numerous cell type in some to almost complete disappearance in others.

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A COLORIMETRIC METHOD SPECIFIC FOR DEHYDROISOANDROSTERONE AND ITS APPLICATION FOR QUANTITATIVE ESTIMATION IN PURE SOLUTIONS AND IN URINARY EXTRACTS[1]

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In 1942 the author observed that heating alcoholic aliquots of dehydroisoandrosterone with sulfuric acid produced a blue color which appeared to be unique for this steroid. Discherl and Zilliken reported similar observations (1943) and indicated work in progress for quantitative determination. However, no further report appeared and the author therefore undertook a study of various factors for quantitative determination in pure solution and in urinary extracts. A preliminary report on pure solutions has been presented (1948). Nielsen (1948) made a similar report almost simultaneously (1948) and published (1948) a method for quantitative use on pure solutions, which is nearly identical in detail with the method developed by the author. Nielsen applied his method to spectrophotometric measurement and with aliquots well above those employed by the author, who developed his method for use with a photoelectric colorimeter (Evelyn) with a workable range of 15 to 125 μg. of dehydroisoandrosterone. However spectrophotometric analysis was made with dehydroisoandrosterone and androsterone over the range 320 to 800 mμ. The absorption curves obtained with 75 μg. aliquots were similar to those reported by Nielsen, who employed aliquots of 200 μg. giving maximum absorption at 600 mμ for dehydroisoandrosterone and negligible absorption at this wave length for androsterone. From 320 to 500 mμ these steroids gave nearly parallel absorption. For other steroids absorption studies were made with a series of filters (Rubicon), 420 to 720, with an Evelyn photoelectric colorimeter.

Like Nielsen, the author studied various factors involved in producing maximum, stable and reproducible color with dehydroisoandrosterone. Other factors and studies in this report include the

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following: specificity of color and structural relations; admixtures of dehydroisoandrosterone and androsterone; effect of further dilution with iso-normal sulfuric acid; recovery from solutions of pure steroids; determination in and recovery from various urinary extracts, and interfering substances; comparison with other methods.

EXPERIMENTAL

Procedure: The procedure adopted is as follows: To an ice cold aliquot of the substance in 0.4 cc. of absolute alcohol, including alcohol blanks, in a test tube, 2 cc. of cold, concentrated sulfuric acid is added. The solution is mixed with a footed stirring rod, heated exactly 2 minutes in a boiling water bath, cooled in an ice bath for 25 minutes, diluted with 8 cc. of 13.2 N sulfuric acid, mixed and cooled again in an ice bath for 10 minutes. The transfer to tubes

TABLE 1. COLOR PRODUCTION WITH CONCENTRATION RANGE OF 10–150 μG. OF DEHYDROISOANDROSTERONE (DA)

Used DA μg.	%T	Density	K×10⁵/μg. of DA
10	91[3] (13)	0.0374	374.
15	86[2] (9)	0.0630	420.
20	80 (9)	0.0969	484.5
25	74 (54)	0.1308	523.2
30	69 (1)	0.1612	537.3
40	61[1] (5)	0.2129	532.2
50	50[3] (72)	0.295	590.
60	41[2] (4)	0.382	636.7
75	31[3] (35)	0.498	664.
100	20[3] (29)	0.683	683.
125	13[2] (7)	0.870	696.
150	9[1] (3)	1.034	689.

Readings 1 hour after dilution in an Evelyn photoelectric colorimeter with filter 580. % T = per cent transmission. Values of % T are the mean values of the number of determinations indicated in ( ). Density = (2 − log T). K = density divided by the μg. of the aliquot.

for colorimetry and read in about 60 minutes after final dilution, using Rubicon fiilter 580 in an Evelyn photoelectric colorimeter, and evaluate against a transmission or density calibration curve.

Spectrophotometrically maximum color is obtained with 600 mμ, but with Rubicon filters in an Evelyn photoelectric colorimeter filter 580 gives higher density than filter 600. The diluent employed is 13.2 N sulfuric acid which produced maximum density as determined with 25, 50 and 100 μg. aliquots of dehydroisoandrosterone. Normality concentration was employed to favor precision in concentration of the diluent. Absolute ethanol was found most favorable as solvent in color development; 95% ethanol and absolute methanol gave lower color values and concentrated sulfuric acid alone on evaporated and dried aliquots produced colors with deviation from Beer's law greater than with absolute ethanol. Other solvents were found to be unsuitable. The color produced attains maximum density in about 1 hour

after dilution, deepens slightly up to about 8 hours after dilution, but fades appreciably on standing over night.

Relation to Beer's law and evaluation of color density: Table 1 presents data of composite values for many determinations over the range of 10 to 150 μg. of dehydroisoandrosterone. The percentage transmission (% T) values are the mean of the number indicated in the adjacent parentheses. The value of K is density per μg. of the substance. It is obvious that the practical range in aliquot size for use with the Evelyn photoelectric colorimeter is from about 15 to

TABLE 2. ABSORPTION AND COLOR WITH VARIOUS STEROIDS

	%T		Color
Steroids Related to Androsterone	50 μg.	100 μg.	
Dehydroisoandrosterone	50[3]	20[3]	blue
Dehydroisoandrosterone acetate*	50	22[2]	blue
Isoandrosterone	95	88[2]	pink
Androstanediol-3β,17α	95	91[1]	pink
Δ⁵-Androstenediol-3β,17α	85[3]	72[1]	brown.-yellow
Δ⁴-Androstenedione-3,17	36	7	purplish-pink
	39	10[2]	(unstable)
Testosterone	55	21	purplish-pink (unstable)
Cis-Testosterone	34	12[3]	purplish-pink (unstable)
Testosterone propionate	61	32	purplish pink (unstable)
17-Methyl Testosterone	93[2]	84[3]	yellow
17-Ethinyl Testosterone	93	79[2]	yellow
Steroids Related to Estrone			
Estrone	91[3]	85[3]	yellow & gr. fluor.
Ethinyl Estradiol	93	87[2]	brilliant pink and gr. fluorescence
Steroids Relate to Corticosterone			
Desoxycorticosterone acetate	34[3]	10[2]	purplish-pink (unstable)
Pregnenolone	96[2]	91[1]	very light yellow

All values are the mean of 2 or more determinations.
* Used 60 and 120 μg. equivalent to 52.4 and 104.8 μg. of free steroid, respectively.

125 μg. of dehydroisoandrosterone. The value of K appears to approximate a stable value with increase in concentration. Thus, Beer's law does not hold strictly, especially for low concentrations, and for evaluation it is desirable to employ a calibration curve. Since production of the characteristic blue color appears to be affected by various influences, it is desirable to run aliquots of standards along with unknowns.

Specificity of color and relation to structure: A variety of steroids were examined with regard to relation to structure, color and absorption. Since filter 580 is employed for dehydroisoandrosterone the values obtained with this filter are of primary importance for comparison. Table 2 records values for steroids whose transmissions were less than 90% for 100 μg. aliquots. Other steroids examined with 100 μg. aliquots, giving above 90% transmission and producing light

yellow or no color were the following, % transmission in parentheses: Androsterone (95), 17-methyl-androstanediol-3β,17α (93), 17-methyl-Δ^5-androstenediol-3β,17α (96), 17-ethyl-Δ^5-androstenediol-3β,17α (94), alpha estradiol (93[1]), estradiol dipropionate (92), estriol (95), progesterone (99[2]), cholesterol (93[3]), Δ^4-cholestenone-3 (99[2]), bile acids: cholic acid (97), lithocholic acid (97), desoxycholic acid (3,12-di-OH) (98[3]), 3,12-di-OH-7-keto-cholanic acid (99), 3,7-di-OH-12-keto cholanic acid (98), 3, 12-di-keto cholanic acid (100), 3,6-di-keto cholanic acid (99), 3-keto-6-OH-cholanic acid (99), 7,12-di-keto-3-OH-cholanic acid (100), 3,7,12-tri-keto cholanic acid (99[2]).

Admixtures of androsterone and dehydroisoandrosterone: Although color density of androsterone with filter 580 is negligible compared to that of dehydroisoandrosterone, in admixtures of these

TABLE 3. RECOVERY OF DEHYDROISOANDROSTERONE FROM MIXTURES OF DEHYDROISOANDROSTERONE (DA) AND ANDROSTERONE (A)

μg. employed of (A) and (DA)		Ratio of (A) to (DA)	Recovery of (DA) per cent
25	25	1 : 1	96.3
50	50	1 : 1	95.2
100	100	1 : 1	91.0
25	50	1 : 2	96.3
50	100	1 : 2	94.0
25	100	1 : 4	100.0
50	25	2 : 1	89.8
100	50	2 : 1	94.5
100	25	4 : 1	83.3

Percentage recovery values relate to values obtained with solutions of pure dehydroisoandrosterone.

steroids in which androsterone concentration is high, or greatly predominates, color density of dehydroisoandrosterone is significantly depressed. This implies low values for dehydroisoandrosterone in nonfractionated neutral steroids if appreciable androsterone is present. This relationship is indicated in Table 3.

Dilution with iso-normal sulfuric acid: To test the stability of the color by dilution without changing the acid concentration a series of different aliquots of dehydroiosandrosterone, between 80 and 100 μg., were determined in the usual way and subsequently diluted 1:2, 1:3, 1:4, and 1:5 with 18.5 N sulfuric acid in the cold. This diluent is approximately isonormal to the original colored solution and its use avoids heating effect due to dilution. However, the color was not diminished in proportion to dilution, but an anomalous and unexplained increase in color density was produced amounting to an average increase of 18, 24, 31, and 36% for the respective dilutions indicated above. Hence, a color too high for reading in the photoelectric colorimeter may not be diluted to bring it within the practical reading range.

Recovery of dehydroisoandrosterone added to fractions of digitonized ketonic fractions of urinary extracts: Digitonin fractionation

was done according to the method of Frame (1944). When fractions were thoroughly washed and subsequent alcoholic aliquots were at boiling water temperature, usually, but not invariably, satisfactory recovery of dehydroisoandrosterone could be obtained. Examples are indicated in Table 4. Whenever the characteristic blue color for dehydroisoandrosterone was obtained, recovery values were usually nearly quantitative. If pink or purplish colors were obtained with urinary extracts recovery was usually low, but on various occasions considerably too high. It was found that pyridine is not an interfering

TABLE 4. RECOVERY OF DEHYDROISOANDROSTERONE (DA) ADDED TO β FRACTIONS OF DIGITONIZED KETONIC URINE EXTRACTS

Specimen*	Aliquot cc.	DA added μg.	DA found μg.	DA recovered μg.	% of added DA recovered
1	0.05	0	36		
	0.05	50	86	50	100
2	0.10	0	72		
	0.10	25	98	26	104
3	0.05	0	24		
	0.05	50	75	51	102
	0.10	0	50		
	0.10	25	76	26	104
4	0.20	0	38.4		
	0.20	25	62	23.6	94.4
	0.20	50	90.7	52.3	104.6

The specimens employed were alcoholic solutions of the digitonin precipitable fraction of ketonic fractions of urinary extracts from adrenal tumor cases.

factor, since 0.1 N concentration of pyridine employed in 0.4 cc. alcohol aliquots containing 60 μg. of dehydroisoandrosterone gave the same values as controls in alcohol without pyridine. When alcoholic aliquots containing 25 and 50 μg. of dehydroisoandrosterone and from 50 to 200 μg. of digitonin were tested against controls without digitonin a marked qualitative color change occurred, blue changing towards purplish pink, and quantitatively color density was enhanced by about 40% with 50 μg. to about 80% with 200 μg. of digitonin, although the latter produces but slight color by itself. This may possibly account for some high recovery values, but no satisfactory explanation is at hand for low recovery values. The color is sensitive to the presence of oxidizing agents. Thus, selenious, selenic, or telluric acid employed in solution in concentrated sulfuric acid abolishes the blue color and reduces absorption almost completely compared to controls and blanks.

Comparison of assay values with other methods: Table 5 presents data for 3 different sets of specimens and comparison of the present method with 3 others less specific for dehydroisoandrosterone. It may be noted that results are practically identical for pure solutions,

but in cases of urinary extracts values by the present method tend to be lower than by the other methods, a result to be anticipated by virtue of its greater specificity. Specimen 3 compared with specimen 4 reflects loss of dehydroisoandrosterone in the process of acid hydrolysis. Hansen has previously reported (1949) an assay comparison for 24 hour dehydroisoandrosterone values of several adrenal tumor cases.

The spectrophotometric method of Nielsen (1948) applied to pure solutions of dehydroisoandrosterone and read in an Evelyn photo-

TABLE 5. COMPARISON OF DEHYDROISOANDROSTERONE (DA) ASSAY VALUE WITH OTHER METHODS

Specimen†	Method*			
	1	2	3	4
	mg. DA	mg. DA	mg. DA	mg. DA
1	1.027	0.910		0.965
2	1.912	2.010		1.910
3	1.970	1.51	1.53	1.51
4	5.33	5.43	4.81	4.60
5	8.43	7.90	8.83	6.28
6	6.69	7.10	6.36	4.18

* Methods: (1) Holthorff and Koch's adaptation (1940) of Zimmermann's method for 17-ketosteroids (1935), slightly modified by the author (1943) and unpublished; (2) is the method of Pincus (1943) employing $SbCl_3$ as color reagent; (3) is a modified Pettenkofer procedure for the determination of dehydroisoandrosterone (Hansen 1949); and (4) is the present method.

† Specimens: (1) and (2) represent 2 different alcoholic solutions of dehydroisoandrosterone, values given in mg. per cc.; (3) and (4) represent fractions of digitonized ketone fractions of 24 hour normal urines with addition of an equal amount of dehydroisoandrosterone to each, in (3) the dehydroisoandrosterone was added before acid hydrolysis, in (4) after hydrolysis, values given in mg. per 5 cc.; (5) and (6) represent fractions of digitonized ketonic fractions of urinary extracts of 2 specimens of an adrenal tumor case, values given as mg. per 5 cc.

electric colorimeter with filter 580 gave nearly identical results with the present method when 37.5% sulfuric acid by volume (about 13.8 N) was employed as diluent. However, when 25% sulfuric acid (about 9.4 N) was used as diluent, a second alternative suggested by Nielsen, color density was much lower, reaching maximum value in about an hour after dilution, and deviation from Beer's law was more marked with aliquots above 100 μg.

DISCUSSION

Only dehydroisoandrosterone and isoandrostane-6-ol-17-one were stated by Discherl and Zilliken (1943*e* to react positively by their qualitative procedure. The latter substance was not at hand and the former is the only steroid so far examined by us which produces a stable blue color, and thus appears to be unique in this respect. The acetate produces the same color quantitatively in terms of free steroid.

The 4 steroids androstenedione, trans and cis testosterone, and corticosterone acetate produced similar colors qualitatively and quantitatively, with some similarity to that produced by dehydroisoandrosterone, giving maxima with filter 580 and quite high density, but purplish pink by reflected and blue by transmitted light, unstable, with fluctuating fluorescence, and with much fading of color and fluorescence on standing overnight. This is in marked contrast to the stable blue color and only slight fluorescence with dehydroisoandrosterone. Discherl and Zilliken (1943) state that androstenedione and corticosterone gave negative reaction by their procedure. All other steroids tested produced qualitatively quite different colors, having low color density with filter 580 and absorption maxima elsewhere in the spectrum.

Androstenedione, testosterone and desoxycorticosterone, having closest color relationship to dehydroisoandrosterone, have in common a 3-keto and Δ^4 unsaturation. Trans or cis direction or esterification of OH on C_{17}, reduction of the carbinol on C_{17} to carbonyl, or substitution of OH with $COCH_2OH$ change color or absorption but little. On the other hand, progesterone and Δ^4 cholestenone-3, with the same structure at C_3 and C_4, and substitution on OH on C_{17} with acetyl and the cholesterol side chain, respectively, produce almost no color at all. Substitution of H on C_{17} with methyl or ethinyl in testosterone produce a marked diminution of color. Corresponding substitution with methyl or ethyl in Δ^5-androstene-5β,17α-diol has a similar effect. Changing direction of OH of androsterone on C_3, as in isoandrosterone, or producing the corresponding 3β,17α-diol changes the color only to a minor degree, but introduction of Δ^5, unsaturation produces the unique color of dehydroisoandrosterone. This would also account for the positive reaction claimed for isoandrostane-6-ol-17-one if dehydration occurred between C_5 and C_6. Further unsaturation, as in androstenedione produces further enhancement of color. Thus, the most characterizing structural features appear to be unsaturation on C atoms 3, 4, and 5 and substitution of H and OH on C_{17}.

This method is more specific for dehydroisoandrosterone than any others. It is not applicable in the presence of relatively large amounts of androsterone and androstenediol. since under such conditions androsterone depresses the color for dehydroisoandrosterone and androstenediol itself produces significant absorption. Nor can it be used in the presence of androstenedione, which, although it produces a different color than dehydroisoandrosterone, has maximal color density with filter 580 and the density is appreciably higher than that for dehydroisoandrosterone. In general, it is definitely not reliable unless the typical blue color is obtained. Hence, its use is restricted to pure solutions and to extracts suitably fractionated and purified. Thus, ketone fractionation must be followed by digitonization or some other suitable procedure to remove interfering ketone bodies.

SUMMARY

A photoelectric colorimetric method has been developed for quantitative determination of dehydroisoandrosterone, applicable to pure solutions and to suitably fractionated urinary extracts. Limitation of applicability to urinary extracts and other factors which affect the color are discussed.

The method is based on a modified Kober reaction, producing a unique, stable, blue color of high density, by heating alcoholic aliquots with concentrated sulfuric acid in a boiling water bath, addition of suitably diluted sulfuric acid for final color development, followed by reading in an Evelyn photoelectric colorimeter, and evaluation against a calibration curve.

Specificity and structural relations are discussed on the basis of testing a variety of different types of steroids. Specificity is nearly unique for dehydroiosandrosterone.

Comparison with similar and other less specific methods is presented on the basis of determinations of dehydroisoandrosterone in pure solution, recovery after addition to fractionated urinary extracts, and determination in fractionated extracts of normal and pathological urines.

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THE EXCRETION OF POSTERIOR PITUITARY ANTIDIURETIC HORMONE IN THE URINE AND ITS DETECTION IN THE BLOOD[1]

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THE EXPERIMENTS of Gilman and Goodman (1937) clearly demonstrated in rats that an effective osmotic stimulus such as dehydration or the administration of hypertonic saline solution could cause the secretion of posterior-pituitary antidiuretic hormone which was detected after its excretion in the urine. These results have been confirmed by other investigators using rats, cats and dogs (Boylston and Ivy, 1938; Ingram, Ladd and Benbow, 1939; Hare, Hickey and Hare, 1941). Walker (1939) who performed most of his experiments with rabbits did not agree with the other workers. The experiments of Gilman and Goodman were the first in which the urinary hormone under investigation was secreted by the gland *in situ*. Earlier, as well as later experiments revealed that oxytocic, pressor or antidiuretic activity appeared in the urine of various mammals after the injection of posterior-pituitary extracts which usually had to be administered in large doses intravenously (Dale, 1909; Heller and Urban, 1935; Jones and Schlapp, 1936; Heller, 1937; Larson, 1938, 1939; Hare, Hickey and Hare, 1941).

The form in which antidiuretic hormone is secreted by the posterior pituitary and in part excreted subsequently by the kidneys is unknown. It may or may not be similar to the stored secretion of the gland. It is known that press-juice of the fresh posterior pituitary appears to contain one or more large protein-like molecules with which all the active principles are associated (Rosenfeld, 1940; Ham and Rosenfeld, 1942). van Dyke, Chow, Greep and Rothen (1942) were able to extract from the bovine gland a homogeneous protein containing the active principles in constant amounts and in a ratio identical with that in fresh or acetone-desiccated posterior lobes. The molecular weight of the pure protein was calculated to be about 30,000 from its sedimentation-constant in the ultracentrifuge. On the other hand, highly active "fragmented" principles can be extracted

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from the gland by rather drastic methods. Presumably these were present in the boiled press-juice of posterior pituitary which was subjected to ultracentrifugation by Rosenfeld (1940) and by Ham and Rosenfeld (1942). Sedimentation of active principles was greatly reduced in acidified boiled press-juice in comparison with fresh press-juice. These authors also reported that there was scarcely any sedimentation of the active principles in the commercial extracts, Pituitrin, Pitressin and Pitocin, whereas a striking concentration of the various activites in the bottom fraction of the ultracentrifuge tubes was observed with fresh press-juice.

There continues to be controversy concerning the dialyzability of separated or mixed active principles. Collodion or cellulose sausage casing has been used as a semi-permeable membrane. Although Gilman and Goodman concluded that antidiuretic activity does not diffuse through Cellophane (cellulose) this finding has been both supported and denied. Earlier work with collodion membranes and later work with cellulose leads to the conclusion that the active principles of commercial extracts of the posterior pituitary may diffuse quite readily through such membranes (e.g. see Donaldson, 1947). The active principles of fresh saline extract (Ham and Landis 1942) or of pure posterior-lobe protein (van Dyke *et al.*, 1942) either diffuse slowly or not at all.

The present experiments were performed as a first step in the characterization of urinary antidiuretic hormone secreted by the intact posterior-pituitary gland. If the antidiuretic hormone in the urine did not undergo sedimentation in the ultracentrifuge, this finding would support the view that the secreted hormone has a low molecular weight comparable to that of the fragmented principles which have been isolated from posterior-pituitary extracts. Supplementing experiments with ultracentrifuged specimens of urine, the dialyzability of urinary antidiuretic hormone also was studied. Lastly, the effect of osmotic stimulation of the cerebrum on the antidiuretic activity of venous blood from the head was investigated.

METHODS

All the assays for the antidiuretic principle were performed in unanesthetized, trained female dogs. Dr. R. C. De Bodo kindly lent us three female dogs with marked diabetes insipidus owing to diencephalic lesions interrupting the supraoptico-hypophysial tracts. The other three dogs were normal. Water was administered repeatedly by stomach tube so that control volumes of urine, before and after injections, were 5ml. per minute or more. Urine volume was determined every five minutes and principal reliance was placed on changes in urine volume in performing assays. The urine was collected from the bladder through a Malecot four-wing catheter which was left in place throughout an experiment. Collection in each five-minute period was completed by the usual method of injecting air and applying supra pubic pressure. A constant rate of excertion of urine before and after each injection

during assay was sought rather than an arbitrary absolute rate. High rates of urinary excretion are especially important in normal dogs to suppress as completely as possible any secretion of antidiuretic hormone. Excretion of endogenous creatinine was usually determined either in thirty-minute periods or, later, in short periods before and during the action of injected antidiuretic hormone to confirm the belief that antidiuresis was not to be attributed to a change in the rate of glomerular filtration. (The method of estimating the quantity of antidiuretic hormone used by Hare and his coworkers (1945) relied on changes either in the concentration of endogenous creatinine in urine or in the ratio of concentration of exogenous creatinine in urine to that in plasma in dogs with diabetes insipidus.)

All the injections were given intravenously, usually into a vein of the fore-limb. Pitressin was used as a standard both for estimating the sensitivity of each assay-dog and for determining the approximate quantity of antidiuretic activity in unknown samples. For nearly all the experiments, a special sample of Pitressin containing 80 pressor units and 3.8 oxytocic units in each mg. was kindly supplied by Dr. A. C. Bratton of Parke, Davis and Company.

The urine or whole blood used for assay was always obtained from dogs. To ensure the highest possible concentration of antidiuretic hormone in the urine, the donor dog either was dehydrated by the withdrawal of water for 48 to 72 hours, or the dog's osmo-receptors (see Verney, 1947) were stimulated by the intracarotid injection of 0.5M NaCl at a rate of 4 to 8 ml. per minute for as long as 90 minutes. The excreted urine was collected serially and samples of maximum potency were set aside in a refrigerator or freezer after the pH had been adjusted to about 4.0. Dr. Marthe Vogt kindly prepared two animals with carotid cuffs into which the hypertonic saline solution could be injected without anesthesia. In other experiments, intravenous α-chloralose preceded by 2 mg. per kilo of intravenous morphine sulfate was used as an anesthetic. Only catheterized specimens of urine were used.

Dialysis under pressure was performed with the apparatus shown in Figure 1, in which a negative pressure of 225 cm. of water was appliedthrough the external water, W. Visking sausage casing was used as the semi-permeable membrane. The apparatus was mounted in a refrigerated room kept at 4° C. The period of dialysis was 16 to 18 hours. The dialysate is defined as the water and solutes which pass through the cellulose tubing. The material held back is called the impermeate or residue (Ball, 1948; Derx, 1947).

The ultracentrifuge used was described by Chiles and Severinghaus (1938). The rotor holds 10 Lusteroid tubes having an inclination of 45° to the axis of rotation. The total capacity of the rotor is about 60 ml. At 48,000 r.p.m., the centrifugal force at the top of the tubes is about 95,000 times gravity and at the bottom is about 180,000 times gravity. The rotor and its contents were refrigerated before running. At the end of each run, the centrifuge was rapidly decelerated to 25,000 r.p.m. and then allowed to coast to rest. The time required for stopping was about 2.5 hours and the temperature of the rotor was then about that of the room. Only negligible mixing of partially sedimented particles occurs if this procedure is followed (Severinghaus, Levin and Chiles, 1938). Samples from the top, middle and bottom of each tube were removed by aspiration from the menisci with a syringe and long needle. Careful manipulation and the slight density gradient from

the top to the bottom of the tubes prevented appreciable mixing of the fluid. Only the top and bottom fractions were used for assay. The volume of the middle fraction was the largest and that of the bottom fraction was the smallest.

A considerable number of experiments was performed to define the most convenient conditions for demonstrating that material for assay was inactivated like posterior-lobe extracts. A rapid method was devised with thioglycollic acid which was also used by van Dyke *et al.* (1942). Thioglycollate as a freshly-made solution of acid neutralized with 1N NaOH is mixed with urine or water containing antidiuretic hormone so that the final concentration is 0.01 M at pH 7.5. After 2.5 hours at room temperature, only 2 per

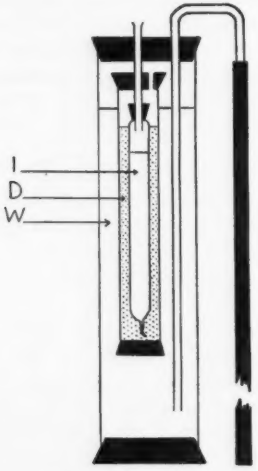

FIG. 1. Apparatus for pressure dialysis. Rubber stoppers were used for closing the two glass tubes and the upper end of the Visking (cellulose) tubing containing the urine which at the end of dialysis is the impermeate, I. The glass tube containing the dialysate, D, is 3.5 cm. in external diameter and 21 cm. long. The outer glass tube is 6.0 cm. in external diameter and 30 cm. long; it contains water, W, through which a negative pressure of 225 cm. of water is applied by means of the rubber tubing after elevation of the apparatus to the proper height.

cent of the activity remain in a solution originally containing 100 m.u. of Pitressin per ml. None of the residual activity of the maximal amount found in the urine (6 m.u. per ml.) can be detected after 40 minutes whether aqueous Pitressin or urine containing excreted hormone be used. One ml. of thioglycollate as 0.01M or 0.05M solution at pH 7.5 can be injected intravenously into normal assay dogs with no evident effect on the diuresis.

RESULTS

Both normal dogs and dogs with diabetes insipidus are very sensitive to intravenously injected antidiuretic hormone in the form of Pitressin. The usual total minimal dose used, and this can ordinarily be detected after injection into a vein of the fore-limb, was 0.25 milli-

unit (m.u.). The range of dosage in unknown solutions was ordinarily equivalent to 0.5 to 1.0 m.u. of Pitressin. (A maximal effect is produced by about 3 m.u. (Hare *et al.*, 1945.)) So far as precision of assay is concerned, 0.25 m.u. could be distinguished from 0.5 m.u. or 0.5 m.u. from 1.0 m.u. It was not always possible to detect the difference in antidiuretic effect of 0.75 m.u. in comparison with 0.5 m.u. or 1.0 m.u. Figure 2 depicts an example of control assays with known dilutions of Pitressin in a normal dog.

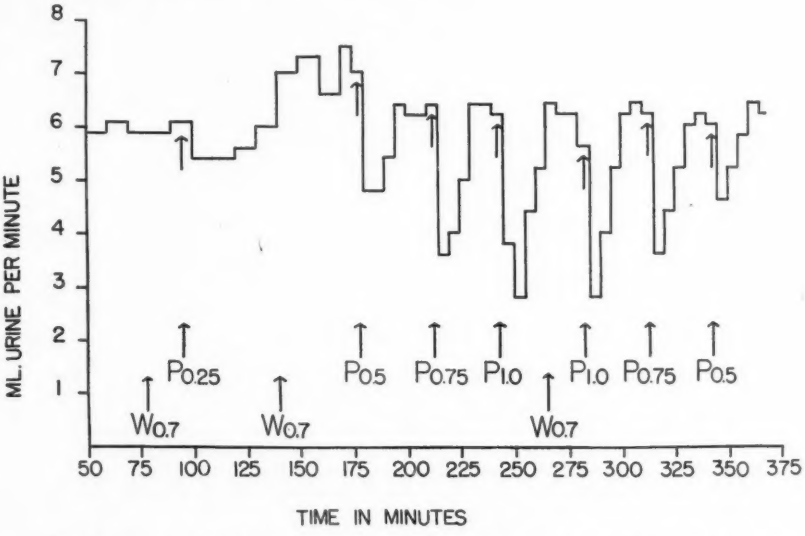

FIG. 2. Assay of antidiuretic hormone in a control experiment. P, series of intravenous injections of Pitressin with each total dose shown as a milliunit or fraction thereof. W, water by stomach tube with doses expressed as fractions of a liter; one liter was also administered at 0 time. Endogenous creatinine excreted before and after each injection varied inconsistently between 0.506 and 0.595 mg. per minute. Normal female dog, weight 18.4 kg.

One milliunit of Pitressin can be clearly distinguished from 0.5 m.u. and the minimum sensitivity of all assays is considered to be of this order. In this particular experiment the antidiuretic effect of 0.75 m.u. is greater than that of 0.5 m.u. and less than that of 1.0 m.u.

The effects of ultracentriguation of urine or Pitressin are summarized in Table 1. Most of the urine specimens were from dogs in which the secretion of antidiuretic hormone was stimulated by the intracarotid infusion of 0.5M NaCl. Sedimentation of the antidiuretic activity present occurred in all the specimens of urine except one which had been boiled 3 minutes (experiments A, B, C, D and E). It was anticipated that heating would liberate an active fragment which would not undergo sedimentation. However, one specimen of urine, and a control aqueous solution of Pitressin were just brought to boil-

TABLE 1. THE ULTRACENTRIFUGATION OF URINE OR DIALYSATE OF URINE CONTAINING ANTIDIURETIC HORMONE OR OF AQUEOUS SOLUTIONS OF PITRESSIN

Expt.	Specimen[1]	Ultra-centrifug.	No. of assay expts.	Potency of fractions in tubes after ultracentrifug.	
				Top	Bottom
		hrs.		m.u./ml.	m.u./ml.
A	Urine D	6	2	0.25	0.75
B	Urine O	6	2	0	1.0
C	Urine O	6	1	0	2.0
D	Urine O	6.5	3	0.5	10.0
	Urine to boil.	6.5	3	1.0	12.0
	Pitressin	6.5	2	1.0	6.0
	Pitr. to boil.	6.5	2	1.5	6.0
E	Urine O	6.5	2	3.0	10.0
	Urine boiled 3 min.	6.5	3	2.5	2.5
F	Urine OI	5.5	3	2.0	5.0
	Urine OD	5.5	3	0.25	0.25
G	Urine OI	4.5	2	0.4	10.0
	Urine OD	4.5	2	2.0	2.0
H	Urine OD	6.0	2	0.5	0.5
	Urine OD mixed with equal vol. of inactive urine.	6.0	2	0.25	0.25

[1] Urine D: Urine of a dog dehydrated 51 hours.
Urine O: Urine of various individual dogs after stimulation of osmoreceptors by intracarotid injection of 0.5M NaCl.
Urine OI: Impermeate of a urine O at end of dialysis.
Urine OD: Dialysate of a urine O at end of dialysis.

ing and promptly chilled. Both the hormone of urine and the dilute aqueous Pitressin still underwent sedimentation (experiment D). On the other hand, the boiling of another specimen of urine was continued for 3 minutes. As a result, there was a loss of activity and a failure of the surviving hormone to undergo sedimentation (experiment E). The special sample of Pitressin was used in experiment D. Another experiment with a diluted commercial sample of Pitressin is not recorded in Table 1. In this experiment there was no sedimentation of antidiuretic activity and it may be concluded that the antidiuretic activity of different lots of Pitressin may or may not be sedimentable in the ultracentrifuge.

A number of control experiments clearly showed that a fraction of the antidiuretic activity of urine dialyzes during a period of 16 to 18 hours at 4°C. A comparison was made of the sedimentation of the antidiuretic activity of the impermeate and the dialysate in two experiments (F and G). As would be expected, the active material in the dialysate could not be concentrated in the bottom layer of the ultracentrifuge tubes. The hormone held back by the membrane, the

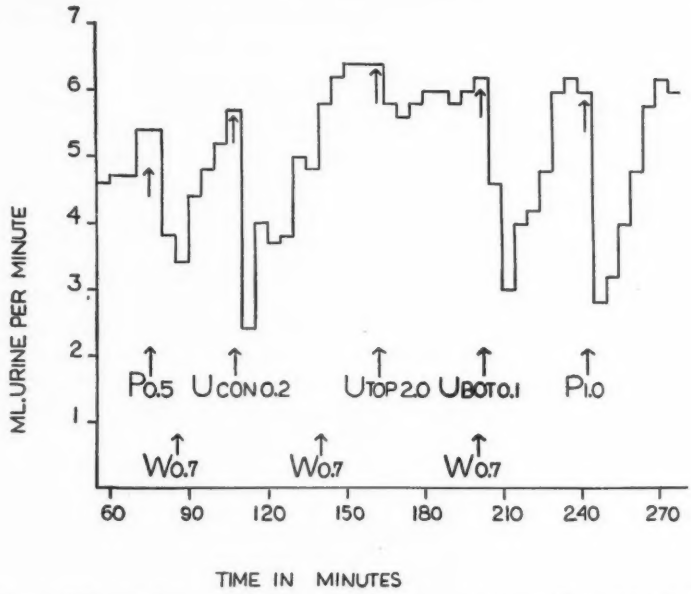

FIG. 3. Effect of ultracentrifugation on the impermeate (or residue) of the dialyzed urine of a dog in which the osmoreceptors had been stimulated by the intracarotid injection of 0.5M NaCl (experiment G of Table 1). P, 0.5 or 1.0 m.u. of Pitressin. U con., intravenous injection of equivalent of 0.2 ml. urine *without* ultracentrifugation; U top, top layer of ultracentrifuged urine, 2.0 ml.; U bot., bottom layer of ultracentrifuged urine, equivalent of 0.1 ml. injected intravenously. W 0.7, 0.7 l. water by stomach tube in addition to 1.0 l. at −3 minutes. Excretion of endogenous creatinine per 30 minutes ranged from 24.0 to 24.8 mg. Assay dog: female with diabetes insipidus weighing 19.0 kg.

The water-clear impermeate of the dialyzed urine contained about 5 m.u. of antidiuretic activity per ml. After ultracentrifugation, the top fraction contained less than 0.25 m.u. per ml. whereas the bottom fraction contained about 10 m.u. per ml. indicating that sedimentation of antidiuretic activity had occurred.

impermeate, unquestionably underwent sedimentation. An example of the assay of the impermeate of experiment G is shown in Figure 3. An assay of the dialysate is shown in Figure 4.

The ease with which oxytocic or vasopressor (antidiuretic) posterior pituitary fractions can be adsorbed is well known; differential adsorption has been used both for separating and for purifying these fractions. Therefore, it is appropriate to inquire whether experimental evidence can be offered against the view that the material undergoing sedimentation is an inert substance of high molecular weight to which the antidiuretic fraction is adsorbed. Such evidence is offered in the experiment of Figure 5 (experiment H of Table 1). Control morning urine of a dog with a carotid loop was secured by catheterization and found to contain no detectable antidiuretic hormone. Five-tenths molar NaCl was then infused into the carotid artery, urine was col-

lected at intervals, and the specimen richest in antidiuretic hormone (6 m.u. per ml.) was dialyzed to secure non-sedimentable antidiuretic activity. A mixture of equal volumes of a portion of the dialysate and of the morning specimen of urine as well as a specimen of the dialysate were ultracentrifuged for 6 hours at 48,000 r.p.m. There was no sedimentation of antidiuretic activity in either set of tubes. In the two assay experiments, one of which is shown in Figure 5, there was excellent agreement and all the antidiuretic activity in either set of tubes could be accounted for. The inert urine used is believed to have contained a much higher concentration of natural constituents than urines rich in sedimentable antidiuretic hormone after the infusion of 0.5M NaCl into the carotid. Such an experiment speaks against the hypothesis that the hormone excreted into urine becomes adsorbed on a substance which undergoes sedimentation in the ultracentrifuge. It is planned to repeat such experiments with normal urine samples which vary in pH and concentration of solutes.

In experiments C, D and E, as well as in other experiments either

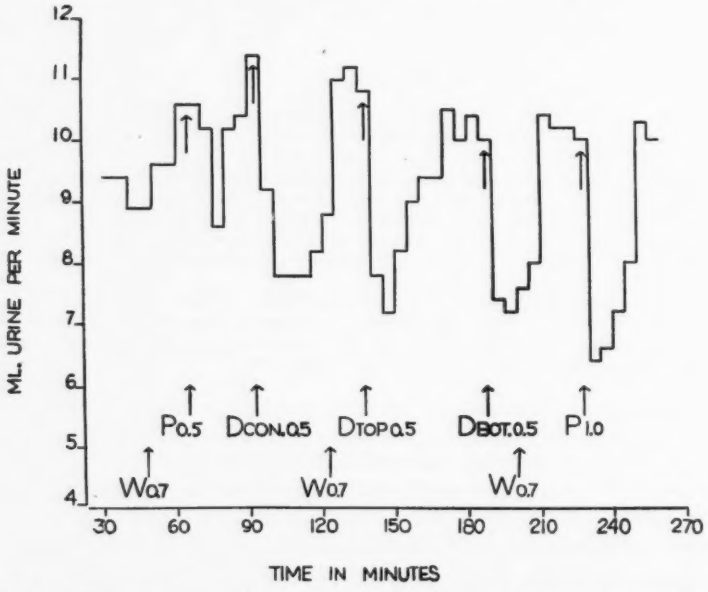

FIG. 4. Effect of ultracentrifugation of the dialysate of the same urine described in fig. 3. P, 0.5 or 1.0 m.u. of Pitressin. D con., 0.5 ml. of dialysate without ultracentrifugation. D top, top layer of ultracentrifuged dialysate, 0.5 ml.; D bot., same dose of bottom layer of ultracentrifuged dialysate. W 0.7, 0.7 l. of water by stomach tube in addition to 1.0 l. at −3 minutes. Excretion of endogenous creatinine per 30 minutes ranged from 27.4 to 28.5 mg. except for determination at 30 to 60 minutes when 30 mg. were excreted. Assay dog: normal female weighing 20.0 kg.

The dialysate contained antidiuretic activity derived from the urine; as would be expected, this activity did not undergo sedimentation in the ultracentrifuge.

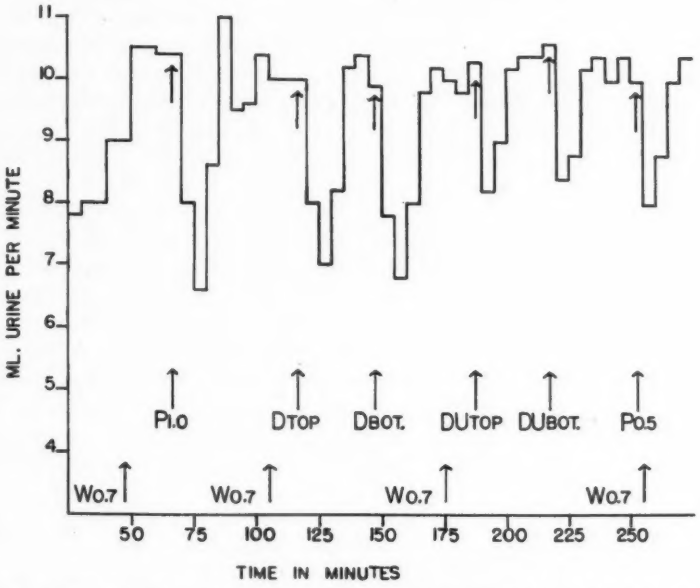

FIG. 5. Effect of ultracentrifugation on a mixture of normal inactive urine and active dialysate from urine rich in antidiuretic hormone (experiment H of Table 1). The dialysate was secured after dialysis under pressure for 16 hours at 4° C. and contained 0.5 m.u. per ml. D top and D bot., injection of 2 ml. of top and bottom layers respectively of ultracentrifuged dialysate. Equal volumes of dialysate and hormone-free control urine of donor dog before infusion of 0.5 M NaCl were mixed, allowed to stand 30 minutes, and then ultracentrifuged: DU top and DU bot., injection of 2 ml. of top and bottom layers respectively of mixture. P, 1.0 or 0.5 m.u. of Pitressin. W 0.7, 0.7 l. of water by stomach tube in addition to 1.0 l. at 0 minutes. Excretion of endogenous creatinine before and after injections ranged from 0.307 to 0.347 mg. per minute. Assay dog: female with diabetes insipidus weighing 18.0 kg.

There was no sedimentation of antidiuretic activity either in the dialysate or after the latter had been mixed with normal urine containing no hormone. Therefore, there was no evidence of adsorption of the active fragment on an inert sedimentable constituent of urine.

with active urine or Pitressin diluted in isotonic saline, the antidiuretic effect was completely abolished by the addition of thioglycollate to a concentration of 0.01M at pH 7.5 provided that the mixture was kept at room temperature for 40 minutes.

Antidiuretic hormone in the venous blood of dogs which were receiving powerful osmotic stimulation of the cerebrum was also investigated. Previously, Hare, Hickey and Hare (1941) reported an experiment in which no antidiuretic activity could be detected in the external jugular blood (less than 1 m.u. per 100 ml.) of a dog although sufficient hormone was being secreted to cause an easily recognized antidiuresis. On the other hand, Melville (1937) concluded that dog's blood (normally hydrated dogs?) contains about 10 m.u. per 100 ml.

and that human blood may contain this concentration or higher concentrations, or may contain no detectable hormone (e.g., normal pregnancy). Perhaps because of the strong osmotic stimulus of intracarotid hypertonic saline, an antidiuretic effect was readily demonstrated in external jugular blood of dogs as shown in Figure 6. The concentration present appeared to be about 10 m.u. per 100 ml. of blood during stimulation of the osmoreceptors whereas no antidiuretic hormone was detected in the control sample. Similar results were obtained in five different experiments. In one experiment, femoral-vein blood contained no appreciable hormone during stimulation of the osmoreceptors. There was no evident antidiuretic effect from the small amount of heparin which was mixed with all the specimens. Since only a fraction of the external jugular blood is derived from the cerebral vessels, two other experiments were performed with blood collected from the internal jugular. A diagram of such an experiment in a heparinized dog is shown in Figure 7. The sample of internal jugular blood richest in antidiuretic hormone (blood C) contained about five

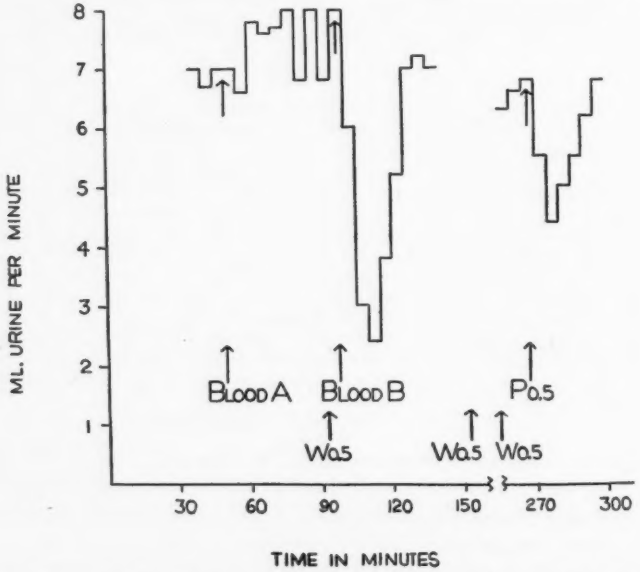

FIG. 6. Release of antidiuretic hormone into the external jugular blood of a donor dog weighing 10.0 kg. after the infusion of 6 ml. per minute of 0.5M (2.93 per cent) NaCl into the carotid artery. Each blood sample was 10 ml. mixed with 2 ml. of isotonic saline containing 5 mg. per cent heparin. Blood A, intravenous injection of control blood; blood, B similar injection of blood collected during the injection of 0.5M NaCl, 33 minutes after the infusion was started. P 0.5, 0.5 m.u. of Pitressin. W 0.5, 0.5 l. water by stomach tube as well as 1.0 l. at −3 minutes. Excretion of endogenous creatinine per 30 minutes ranged from 18.3 to 19.2 mg. Assay dog: normal female weighing 16.5 kg.

Stimulation of the cerebral "osmoreceptors" was followed by the appearance of one m.u. or more of antidiuretic hormone in 10 ml. of external jugular blood.

times as much hormone (50 m.u. per 100 ml.) as the external jugular blood of other experiments. It is unlikely that the possible hypertonicity of the jugular blood during the intracarotid injection of 0.5M NaCl affected the assay dogs. In control experiments in normal or diabetes-insipidus dogs used for assay, the injection of as much as 10 ml. of N NaCl into a vein of the fore-leg caused no antidiuresis.

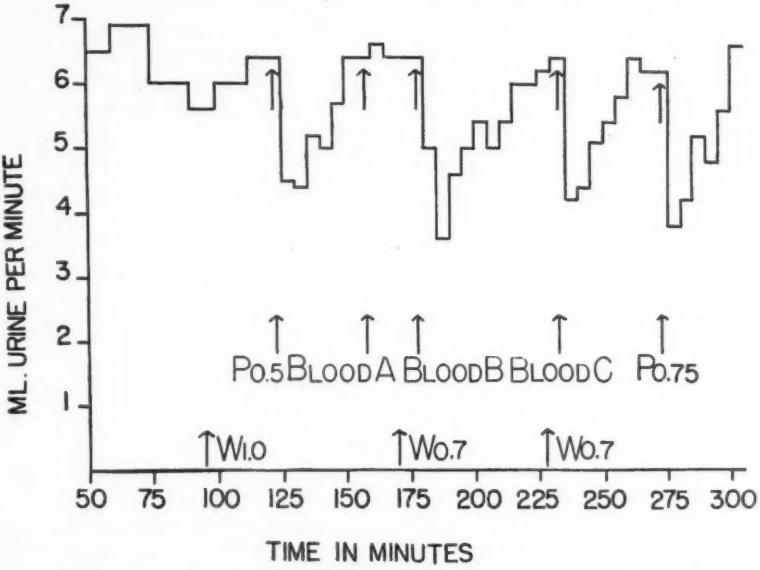

FIG. 7. Release of antidiuretic hormone into the internal jugular blood of a heparinized donor dog weighing 14.0 kg. after the infusion of 6 ml. per minute of 0.5M (2.93 per cent) NaCl into the carotid artery. All blood samples collected from cannula in internal jugular after heparinization. Blood A, intravenous injection of 2 ml. of control blood; blood B, similar injection of 2 ml. of blood collected 25 minutes after infusion started; blood C, injection of 1 ml. of blood collected 35 minutes after infusion started. P, Pitressin standard as 0.5 or 0.75 m.u. W, water by stomach tube as liters; 1 liter was also given at 0 time. Excretion of endogenous creatinine, determined before and after injections, ranged from 0.310 to 0.348 mg. per minute. Assay dog: female with diabetes insipidus weighing 20.0 kg.

The *internal* jugular blood (sample C) contained about 0.5 m.u. of antidiuretic hormone per ml. during stimulation of the cerebral osmoreceptors.

DISCUSSION

The experiments here reported are in harmony with the view that antidiuretic hormone is excreted in the urine of the dog as a large molecule as shown by its sedimentation in the ultracentrifuge. If urine containing the hormone is dialyzed through a cellulose membrane under pressure, a part of the activity, as a smaller fragment escapes through the semi-permeable membrane; however, the impermeate contains the bulk of the hormone and still can be concentrated by the ultracentrifuge. Separation of the active fragment does not occur as

readily as was expected (e.g., with heat). The antidiuretic substance in urine is inactivated in the same fashion as posterior-pituitary extract or Pitressin with thioglycollate. Also, it was found that Pitressin may or may not undergo sedimentation. This may depend on variations in preparation, the method of which is kept confidential by the manufacturer. In the ultracentrifugation experiments of Rosenfeld (1940) and Ham and Rosenfeld (1942), there was very slight sedimentation of active principles in commerical samples of Pituitrin, Pitressin and Pitocin. Many of the present experiments were performed with a specially purified sample of Pitressin which was sedimentable. A clear aqueous solution of this particular sample became turbid on the addition of tricholoracetic acid to a concentration of 5 per cent; certainly this finding suggests the presence of protein.

To what other extent the small amounts of hormone excreted resemble the protein extracted from the bovine neural lobe by van Dyke *et al.* is unknown. It is obviously important to determine whether there is parallel excretion of oxytocic activity undergoing similar sedimentation. Difficulties of assay of small amounts of oxytocic activity in urine have prevented satisfactory progress but it is hoped that a good assay-method can be devised.

The possibility of adsorption of an active antidiuretic principle on an unknown substance of high moleuclar weight cannot be denied. In one experiment, Pitressin was added to urine containing no hormone. Unfortunately, the Pitressin in saline, used as a control, underwent similar sedimentation in the ultracentrifuge. It has already been emphasized that a part of the total activity as the antidiuretic fragment separates from the hypothetical hormone and appears in the dialysate of urine rich in hormone. When this non-sedimentable activity is mixed with normal urine it undergoes no sedimentation in the ultracentrifuge. This is evidence against the view that the fragment is easily adsorbed on an inert sedimentable substance in urine. The experiments reported by Donaldson (1947) have also a bearing on this question. He mixed Pitressin with normal human urine (3.7 per cent solids) and demonstrated that pressor-antidiuretic activity dialyzed from the mixture as readily as from an aqueous solution of Pitressin. This observation speaks against adsorption of antidiuretic hormone in urine although its significance in this regard is perhaps reduced by the single long period of dialysis used (17 hours).

SUMMARY

Urinary antidiuretic hormone secreted by the posterior pituitary of dogs, in response to stimulation of the cerebral osmoreceptors by dehydration or by the intracarotid injection of hypertonic sodium chloride solution, underwent sedimentation in the ultracentrifuge. A fraction of the hormone, believed to represent a fragment of low molecular weight, was separated by pressure-dialysis and could not be

concentrated in the ultracentrifuge whether as a dialysate or after mixing with normal inactive urine. The significance of these findings is discussed.

Antidiuretic hormone could be detected in the jugular blood during powerful stimulation of the osmoreceptors by the intracarotid injection of hypertonic salt solution but was absent from femoral-vein blood. Internal jugular blood contained as much as 50 m.u. per 100 ml.

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RESUMPTION OF FUNCTION OF AUTOPLASTIC ADRENOCORTICAL TRANSPLANTS TO THE DORSAL MUSCULATURE IN RATS

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THE TECHNIQUE of autogenous grafts of adrenocortical tissue in rats is often used when it is desirable to study the function of the cortex without concomitant medullary function. Most investigators have waited a month or more for grafts to regenerate before continuing the experiment (Eversole, Edelmann, and Gaunt, 1940; Wyman and tum Suden, 1942; Weinstein and Schiller, 1949). The report of Ingle and Higgens (1938) that anatomical regeneration begins on the third post-operative day suggested that physiological function is resumed much sooner than had been thought. In order to determine the course of resumption of function of autoplastic adrenocortical transplants to the dorsal musculature, responses to three types of stress, namely, histamine poisoning, exposure to cold, and water intoxication were studied. Although more highly vascularized sites than the skeletal muscles are preferred by many investigators, the muscular site was chosen for this study because its proximity to the operational field for dorsal adrenalectomy makes it the most suitable one for certain experiments. Moreover intramuscular transplants have been employed in numerous previous studies in this laboratory.

METHODS

A total of 214 male albino rats of the Wistar strain were used. The mean weight was 130 ± 30 grams at the time of the tests. Adrenalectomy was performed by the dorsal route under ether anesthesia. The glands were removed together with most of the surrounding fat, trimmed, bisected, and the four halves were placed in pockets in the spinotrapezius and dorsolumbar muscles. The animals were given saline drinking water for three post-operative days, then tap water and standard Purina or Rockland Farm Chow were supplied *ad libitum*.

Histamine acid phosphate (Burroughs Welcome) in a 5 per cent solution was injected intraperitoneally in doses of 10 or 15 mg. per 100 gm. of body weight. The animals were observed for four hours or until death occurred.

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Recovery from shock was taken as the criterion of functioning cortical tissue.

The cold test was administered according to the method of Roos (1943). Animals were placed in an ice box at $2 \pm 2°$ C. and the colonic temperature, Fahrenheit, was recorded at hourly intervals for four hours. The rats were accustomed to insertion of the thermometer into the colon for several days prior to the test. Maintenance of a normal body temperature for four hours was taken as an indication of efficient cortical function.

Water intoxication was caused by giving 6 cc. of water per 100 gm. of body weight by gavage every hour for four hours, after having deprived the animal of food for twelve hours and of water for two hours. The rats were kept in metabolism cages for eleven hours following the first administration of water, and the total volume of urine eliminated during that period was measured. The elimination of those that died was recorded for the survival period of 5 to 7 hours. It was about the same as that of the survivors. Intact rats excreted 90 per cent or more of the total volume ingested.

The criteria of death were cessation of respiration, sensibility, and reflex responses. All animals were autopsied to ascertain the condition of the transplants or the presence of "accessory" cortical tissue. Acessory growth rarely occurred, and when found the animals were eliminated from the series.

RESULTS

In the experimental animals one, two or three, but never four, apparently functional transplants were observed. Gross criteria of viable transplants were signs of vascularity and a rosy red translucency, as contrasted with the chalky white opaqueness of unregenerated grafts.

Histamine tolerance tests. Six intact animals exhibited signs of only slight abdominal pain and apparently recovered within 15 minutes after doses of 15 mg. per 100 gm. body weight of histamine. Five adrenalectomized rats died within 15 to 30 minutes after 15 mg. per 100 gm., 4 to 18 days after operation. Of two that received 10 mg. per 100 gm., one died in an hour (6 days after operation) and the other in four hours (13 days after operation). Death was preceded by general cyanosis, prostration, and convulsions. Autopsy revealed the loss of fat, etc., characteristic of adrenal insufficiency. Twelve rats with transplants tested from 11 to 15 days after operation recovered from shock following doses of 10 mg. per 100 gm. Eleven transplanted rats tested seven days after operation also recovered, six after doses of 10 mg. per 100 gm., and five after 15 mg. per 100 gm. The time of resumption of maximum efficiency of the transplants in protecting aginst histamine was not determined by further quantitation of the dosage but such work is now in progress.

Cold tests. Eight intact rats maintained a normal temperature for the entire period of exposure to cold (see Table 1). They were active throughout the period and shivered visibly after two hours. Seven adrenalectomized rats lived only two hours, their temperature falling rapidly during that time. All sixty-two animals with adrenocortical

TABLE 1. RESULTS OF COLD TESTS; 4 HOURS AT 2° ±2° C.

Description	No. of rats	Days after operation	Average colonic temperature, ° F.				
			0 hrs.	1 hr.	2 hrs.	3 hrs.	4 hrs.
Normal	8		98.5	98.8	98.5	98.3	98.5
Adrenalectomized	7	6	97.4	95.7	<94.0	dead	dead
Transplant	7	7	97.2	96.4	94.8	<94.0	<94.0
Transplant	8	8	96.6	95.6	94.1	<94.0	<94.0
Transplant	6	9	96.8	97.1	96.7	95.8	94.7
Transplant	29	10	98.5	98.7	98.7	98.5	98.6
Transplant	8	12	98.7	98.7	98.8	98.8	98.7
Transplant	4	14	98.1	98.5	98.6	99.1	98.7

transplants tested from 7 to 14 days after transplantation lived for four hours. There was an apparently stepwise progression in the resumption of functional efficiency in protecting against cold. The 7 and 8 day groups may be said to have reacted alike. The transplants afforded maximum protection to this stress by 10 days after operation. In these animals shivering was also observed. Their grafts showed a combination of the rosy red translucency and the chalky opacity described above.

Water intoxication tests. Eleven intact rats excreted over 90 per cent of the ingested water during eleven hours (Table 2). Five adrenalectomized rats excreted only 13 to 15 per cent, and two of these died in 4 and 7 hours respectively. In rats with adrenocortical grafts a stepwise resumption of functional efficiency was not too definite, but the 10 day group showed some improvement over the 6, 7 and 8 day groups, and the mortality was reduced. Only three animals of the 6 to 8 day groups excreted 20 per cent or more of the ingested water, whereas only one of the 10 day group excreted less than 20 per cent. The 6, 7 and 8 day groups were little better than the adrenalectomized rats, excreting only 1 to 24 per cent of the water, with eleven of the

TABLE 2. RESULTS OF WATER INTOXICATION TESTS: DOSE OF WATER—6% OF BODY WEIGHT HOURLY FOR 4 HOURS

Description	No. of rats	Days after operation	No. of deaths	% of ingested water excreted in 11 hours (average)
Normal	11		0	94
Adrenalectomized	5	8–10	2	9
Transplant	6	6	6	13
Transplant	6	7	3	17
Transplant	6	8	2	18
Transplant	8	10	3	23
Transplant	6	12	1	26
Transplant	6	14	2	23
Transplant	9	16	3	30
Transplant	6	18	0	26
Transplant	6	20	1	26
Transplant	6	22	1	30
Transplant	5	24	0	24
Transplant	6	26	1	24
Transplant	9	32–53	0	46

eighteen rats dying in 5 to 7 hours. From ten days on no rat excreted less than 20 per cent of the ingested water, but little improvement in resistance was noted except in a few isolated cases until more than 30 days after transplantation. Only eight of the 58 rats of the 10 to 26 day groups excreted over 30 per cent (31–52%) while the other 50 animals excreted from 20 to 28 per cent. Indeed, no animal, even after 53 days following transplantation, excreted more than 55 per cent, but the 32 to 53 day group was definitely better than all the preceding ones, excreting from 41 to 55 per cent of the ingested water. It is likely that the maximum protection against water intoxication which can be afforded by intramuscular grafts was present by this time, but it is obvious that such grafts are less efficient in this respect than the intact gland. About half of the rats with transplants over 12 days old were subjected to the cold test at least two days before the water intoxication test and were found to be maximally efficient in maintaining their body temperature.

A peculiar phenomenon encountered during the administration of the water intoxication tests, not previously reported in the literature, was the formation of a penal plug in about 80 per cent of the adrenalectomized and transplanted rats. This occurred five or six hours after the start of the procedure. Its consistency was not determined, but it was white and waxy in appearance and sticky to the touch, resembling a vaginal plug. Microscopically it stained as a lipid and no cellular material was observed in it. It may have been a precipitate from a very concentrated urine or a prostatic secretion, but no residue was found in the bladder at autopsy. This condition was not observed in intact animals, and its presence may have been partly responsible for the reduced amount of urine collected. Although the bladder was not distended at autopsy, the abdominal fur in these rats was always soaked with urine indicating a slow dribbling due to partial closure of the urethra. The amount of urine lost by evaporation was probably not appreciable since the collecting funnels were of highly polished metal and the urine was collected under toluene.

In all animals with transplants older than ten days, the grafts were found to be hemorrhagic at autopsy after water intoxication.

DISCUSSION

The results show that some protection against histamine poisoning and exposure to cold is afforded by adrenocortical transplants to the dorsal musculature within seven days after operation and against water intoxication by ten days. Complete protection from a dose of histamine or a cold exposure which would kill an adrenalectomized rat was obtained seven and ten days after operation respectively. Further quantitation was not carried out to test the maximum efficiency of these transplants in protecting against larger doses of histamine or greater degrees of cold so they cannot be compared with the normal glands in this respect, but beginning ten days after operation the rats

with transplants behaved essentially like normal animals when subjected to the considerable stress of the cold test. The maximum protection against water intoxication which the grafts were capable of affording was not obtained until about thirty days after operation and it was considerably less than that offered by the normal gland. Eversole, Edelmann, and Gaunt (1940) tested the efficiency of adrenocortical grafts to various sites after forty-two to fifty days and found that transplants to the intestinal mesentery, kidney, or ovary were the most efficient, but even these animals did not excrete more than 55 to 74 per cent of the ingested water. Their intramuscular grafts were not particularly efficient, but they tested only four such animals.

Greep and Deane (1949) have concluded that the zona glomerulosa in the rat secretes desoxy-corticosteroids, which regulate fluid and electrolyte balance, and that the fasciculata secretes 11-oxy-corticosteroids, which are concerned with gluconeogenesis and resistance to stress. It is possible that in resuming physiological activity, adrenocortical transplants do not secrete all the active principles at the same time or to the same degree. The regenerated fasciculata may supply enough hormone fairly soon after operation for an almost normal resistance to a stress such as exposure to cold, while the glomerulosa is never able to secrete enough for a normal regulation of water balance. Wyman and tum Suden (1937, 1942) found that intramuscular transplants, two to four months after operation, were less efficient than the normal glands in acute emergencies which demand rapid redistribution of body fluids, such as histamine poisoning or intraperitoneal injection of isotonic glucose solution. Weinstein and Schiller (personal communication) report that their intrauterine transplants have an abundant growth of the zona fasciculata but a small glomerulosa.

SUMMARY

Seven days after operation, adrenalectomized rats with autoplastic adrenocortical transplants to the dorsal musculature recover from doses of histamine which would kill adrenalectomized rats (10 or 15 mg. per 100 gm. body weight). Rats with transplants, seven days after operation, survive a four hour exposure to cold (2° C.) which would kill adrenalectomized rats, and by ten days after operation they maintain normal colonic temperatures. Rats with transplants exhibit some resistance to water intoxication by ten days after operation, and the maximum of which they are capable by about the thirtieth post-operative day, but they never recover a normal resistance to this stress.

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FAILURE OF SALT REPLACEMENT THERAPY IN ADRENALECTOMIZED RECENTLY CAPTURED WILD NORWAY RATS[1]

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IN A PREVIOUS paper it was reported that the adrenal glands were much larger in recently captured wild Norway rats than in the domesticated Norway rats from our colony (Rogers, Richter, 1948). These results confirmed the earlier findings of Watson (1907), King and Donaldson (1929), Hatai (1915), and Donaldson (1923). They also confirmed the findings of Donaldson (1923) that an increase in cortical tissue accounts for the entire difference.

In the present investigation we were interested in determining whether the larger size of the adrenals actually indicates that wild rats have a greater need for the adrenal cortical secretions. To do this we compared the effects of salt therapy on domesticated and wild rats after their adrenals had been removed. From earlier experiments it was known that following adrenalectomy most domesticated rats live and thrive on salt therapy alone. It remained to be determined whether the adrenalectomized wild rats would do likewise.

METHODS

The rats were kept in individual cages each of which contained a non-spillable food cup and one or two graduated inverted 100 cc. water bottles. Specially constructed cages were used for the wild rats.

Records were made daily of food and fluid intakes, and weekly of body weights.

The diets used in these experiments were (1) our regular stock diet* and (2) fox chow meal.† Both of these diets contain 1% salt (sodium chloride) in addition to any residual amounts that exist in the other ingredients (such

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* Graham flour 72.5%, casein 10.0%, skim milk powder 10.0%, butter 5.0%, calcium carbonate 1.5% and sodium chloride 1.0%.

† Purina fox chow (with meat meal).

as the butter in our stock diet). For a "salt-poor" diet we used our regular stock diet, with flour replacing the sodium chloride: this diet was estimated to contain a maximum of 0.1% sodium chloride, or 1 mg./gm. For "high salt" diets we used either (1) or (2) above with the salt contents increased to 3% or 4% by adding extra sodium chloride.

In one group the rats were allowed to regulate their own salt intake; in other groups they were forced to take the salt either in their drinking water or in their food, or both. When allowed to regulate their own intake they were given access to a 3% salt solution in a container separate from the one used for their drinking water.

The 71 (35♂ and 36♀) adult wild rats used in these experiments were captured from the alleys and yards of Baltimore and were kept in the laboratory several weeks before being used (Richter, Emlen, 1945). The 70 (44♂ and 26♀) adult domesticated rats that were used as controls came from our albino and hooded colony that has been in existence for over 25 years. The original stock of albinos came from the Wistar Institute; a small number of hooded rats was introduced about 20 years ago from the colony of Dr. E. V. McCollum.

To obtain base lines for food and water intake, records were made daily for 10 to 20 days before adrenalectomy. After adrenalectomy these records were continued until the rats either died or gave evidence of living for a long time. At death all of the rats were searched for adrenal rests or accessory nodules.

The operative technique was essentially the same as was described in an earlier paper (Richter, 1941). To avoid leaving adrenal rests, care was taken not to touch the adrenals themselves at any time during the operation; to avoid leaving accessory nodules the adrenal vessels were clamped as near as possible to the renal arteries and veins.

RESULTS

A. Control Observations

Adrenalectomized Rats (Wild and Domesticated) without Replacement Therapy

In these experiments adrenalectomized wild rats were kept on the "salt-poor" diet without access to a salt solution. Fig. 1A shows a typical food and water intake record of one of the wild rats that died. The ordinates give food intake in grams and water intake in cubic centimeters, the abscissas time in days. The daily food intake of this rat averaged 17 grams for a 10-day period before adrenalectomy. On the 1st day after operation the intake dropped to 9 grams and from then on decreased steadily, reaching zero on the last day of the rat's life. The water intake was similar although on the first post-operative day it did not drop as markedly as did the food intake. From then on, however, it decreased rapidly until the rat died.

Fig. 1B gives the record of a wild rat that did not die after adrenalectomy, and when autopsied was found to have an adrenal nodule. The post-operative food intake dropped to a level approximately half

as high as that present before adrenalectomy. It remained at this level for about 15 days and then increased to its original level. Water intake decreased for 1 day, after which it actually surpassed the pre-operative level. Autopsy performed on the 63rd day disclosed the presence of a small adrenal nodule on the adrenal vein.

Table 1 summarizes the results. Of 13 wild rats, only 2 survived; both of them had adrenal nodules. The 11 rats without nodules lived on the average 8.5 days after adrenalectomy. Of 30 domesticated rats, 28 died; the 2 survivors had nodules. The 28 rats lived on the average 9.5 days after adrenalectomy. For both groups of rats the average

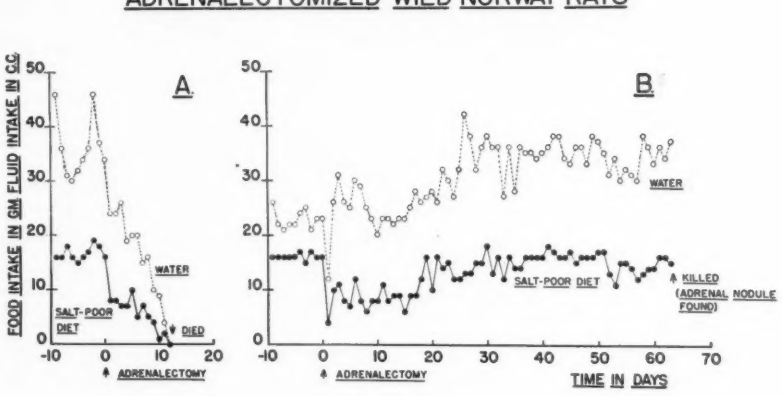

FIG. 1

daily salt intake fell far below an average figure of 577 mg./kg. found for normal rats on the regular stock diet (Richter, 1936).

B. Salt Therapy

Adrenalectomized Rats (Wild and Domesticated) Having Access to a 3% Salt Solution

In these experiments the rats had access to a food cup and two graduated inverted water bottles, one filled with water, the other with a 3% salt solution. Fig. 2A gives a typical record of the food, water, and salt solution intakes of one of the wild rats that showed an increased appetite for the salt solution. This animal increased its daily intake of salt solution from an average of 1.9 cc. for the 10 days before operation to 10.8 cc. after operation, or 468.4%. However, in spite of this increased intake it died on the 17th day. Its food intake showed an initial decrease and thereafter tended to remain fairly constant at a lower level; its water intake showed a small decrease. On the 16th day the food and water intake showed a trend toward normal. The death of the animal on the following day came as a surprise.

Table 2A summarizes the results. It gives the records for two groups of adrenalectomized wild Norways (5 rats on the "salt-poor" diet; 23 rats on fox chow meal) and for one group of domesticated Norways (40 rats on the "salt-poor" diet). Of the 5 wild rats on the "salt-poor" diet, 4 died. The 1 survivor had an accessory nodule. Of the 23 wild rats on the fox chow, 21 died; the 2 survivors also had accessory nodules. Of the 40 domesticated rats only 6 died. One of the 34 survivors had a nodule, leaving 33 or 82.5% that the therapy had kept alive.

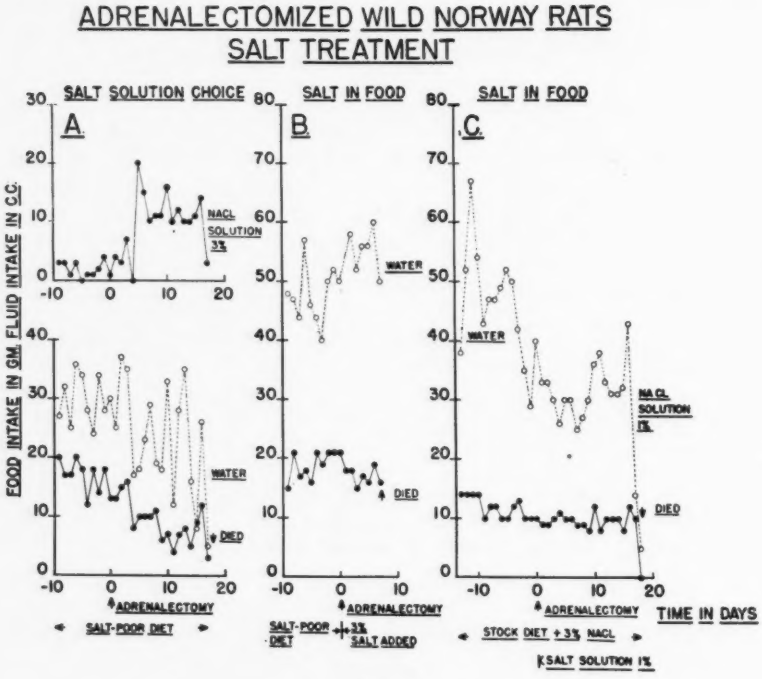

FIG. 2

Table 2B summarizes the observations made on the salt intake of these rats. (Data on rats with nodules have been omitted.) All 4 wild rats on the "salt-poor"diet increased their intake of the salt solution. Their average daily intake increased from 2.1 cc. for the 10 pre-operative days to 9.7 for the last 10 days of life, or 361.9%. During the last 10 days of life their daily salt intake averaged 1029 mg./kg., more than twice the normal average. Despite this increase all 4 died. Their average survival time of 17.8 days as compared to 8.5 days for rats on the "salt-poor" diet shows that the therapy did have some effect.

TABLE 1. UNTREATED ADRENALECTOMIZED RATS
"Salt-poor" Diet

	Wild Norways		Domestic Norways	
	No. of rats	Average daily intake of salt in mg./kg. last 10 days of life	No. of rats	Average daily intake of salt in mg./kg. last 10 days of life
Adrenalectomized	13		30	
Died	11	16	28	34
Survived				
with nodules	2	55	2	72
without nodules	0		0	
Survival time of rats that died (days)	8.5 (2–13)		9.5 (4–19)	

Of the 21 wild rats on the fox chow diet, 15 showed essentially no change in salt intake (7.7%); 6 showed a definite increase (550.0%). The total daily salt intake of the latter group averaged 863 mg./kg. The 15 rats that showed no change in salt intake lived 9.7 days, or just about as long as did the controls; the 6 rats with a large increase lived 25.0 days or about 3 times as long as did the controls.

All 6 of the domesticated rats that died showed an increased intake of salt solution and lived much longer than did the controls. Their survival times averaged 69.7 days; 1 rat lived 166 days. All 33 of the domesticated rats that survived (killed on the average 80 days after adrenalectomy) showed an increased intake of salt solution, on the average 380.0%. For both groups of domesticated rats the total daily salt intake was over twice the normal average.

TABLE 2. ADRENALECTOMIZED RATS WITH ACCESS TO A 3% SALT SOLUTION

A. Number of rats

Strain	Diet	Total	Died	Survived	With nodules	Survived owing to therapy	Per cent
Wild Norway	Salt-Poor	5	4	1	1	0	0
Wild Norway	Fox Chow	23	21	2	2	0	0
Domestic Norway	Salt-Poor	40	6	34	1	33	82.5

B. Intake of salt solution—(Rats without nodules)

Strain	Diet	No. of rats	No. with changes in salt intake	Average daily intake of salt solution in cc.			Average daily intake of salt in mg./kg. for last 10 days of life			Average survival time in days
				10 days before adrenal.	Last 10 days of life	In crease in per cent	From salt solution	From diet	Total	
Wild Norway Rats Dying	Salt-poor	4	4 incr.	2.1	9.7	361.9	996	33	1029	17.8
Wild Norway Rats Dying	Fox Chow	21	15 no significant change	1.3	1.4	7.7	158	54	192	9.7 (3–18)
			6 incr.	1.0	6.5	550.0	627	236	863	25.0 (13–48)
Domestic Norway Rats Dying	Salt-Poor	6	6 incr.	4.9	9.8	100.0	1422	40	1462	69.7 (8–166)
Rats Surviving	Salt-Poor	33	33 incr.	2.5	12.0	380.0	1284	40	1324	

TABLE 3. EFFECT OF SALT THERAPY ON ADRENALECTOMIZED WILD NORWAY RATS

Therapy	Diet		No. of rats		Survival time in days (rats that died)	Average daily intake of salt in mg./kg. last 10 days or less of life (rats that died)			No. of rats that survived	
	Before adrenal.	After adrenal.	Adrenalectomized	Died		From drinking water	From Food	Total	With nodules	Owing to therapy
1% Salt in Drinking Water	Salt-Poor	Salt-Poor	10	10	11.7 (8–17)	969	34	1003	0	0
3% Salt in Food	Salt-Poor	3% Salt Added	12	9	11.2 (5–28)		926	926	2	1
4% Salt in Food	Stock diet +3% salt	Stock diet +3% salt	8	7	14.3 (8–19)	1645	1448	3093	1	0

An explanation of the failure of adrenalectomized wild rats to manifest an increased appetite for the salt solution may be found chiefly on a psychological rather than a physiological basis. The results of previous self-selection experiments in which adult wild rats had access to a variety of purified, or nearly purified, substances showed that, unlike domesticated rats that grow and thrive on their selections, the wild rats die in a relatively short time—usually in 40 days (Richter, 1946). Apparently owing to a high degree of suspiciousness of all new foods or solutions they refuse to take any of the substances offered for choice except vegetable oil, with which they presumably were familiar when they foraged in garbage pails for their food. In marked contrast the domesticated rats usually sampled all of the foods and solutions freely. In the present experiments these same differences appear in the pre-operative records of the salt solution intake. Whereas all of the 39 domestic rats took measurable amounts (over 1.5 cc. per day, the average allowance for evaporation) of the salt solution, only 4 of the 25 wild rats took a comparable amount. The daily records for the individual rats showed that during the 10 days before adrenalectomy very few of the wild rats even sampled the salt solution. It is likely therefore that after adrenalectomy many of them did not sample the salt solution and so did not have the opportunity to experience whether or not the salt solution had a more attractive taste, or whether or not the ingestion of the salt solution made them feel better.

The results of these experiments showed that 40% of the adrenalectomized wild rats voluntarily increased the salt intake to levels well above the normal average; however, despite the ingestion of these amounts of salt they did not survive. It appears that salt alone may not suffice to keep an adrenalectomized wild rat alive; however, the possibility still remains that the rats might have survived had they taken even larger amounts of the salt solution. To settle this question further experiments were undertaken in which the attempt was made to force the rats to take more salt.

Effect of Feeding Adrenalectomized Wild Rats High Amounts of Salt in Drinking Water or Food

In the first series of these experiments all the water the rats had access to contained 1% salt. This forced the rats to take salt to satisfy their thirst. Table 3 summarizes the results. Of 10 adrenalectomized rats that received the 1% salt solution, all died. They survived only a few days longer than did the controls. However, they did not get more salt by this method than they did in the previous experiments in which they regulated their own salt intake.

In the second series 3% salt was added to the "salt-poor" diet. This meant that to satisfy their hunger the rats were forced to take definitely larger amounts of salt. Fig. 2B gives the record of one of

these rats. Adrenalectomy had no detectable effect on the food or water intake. Thus salt therapy appeared to have achieved full replacement. However, on the 7th day, despite these high food and water intakes, the rat died. Table 3 shows that of 12 rats on this diet, 9 died after an average of 11.2 days. Of the 3 that survived, 2 had adrenal nodules. In one no nodules were found, so it apparently had been kept alive by therapy. For the 9 rats that died the daily salt intake averaged 926 mg./kg. which was definitely above the normal average but not much above the average of the rats that regulated their own salt intake. Most of the rats that died gave much the same type of record that was shown in Fig. 2B.

In the third series 3% salt was added to the stock diet to make a total of 4% salt; and immediately after the operation a 1% salt solution was substituted for the plain drinking water. Fig. 2C gives a typical record. After adrenalectomy this animal continued to eat about the same amounts of the high salt food as before; also it drank about the same amounts of the 1% salt solution as it had previously taken of the plain water (the intake had decreased to a lower level several days before adrenalectomy). Like the rat in Fig. 2B this one died a very sudden death at a time when full replacement appeared to be achieved. Table 3 summarizes the results. Of the 8 adrenalectomized rats on this diet, 7 died after an average of 14.3 days; the 1 survivor had an adrenal nodule. The total daily intake of salt of the 7 rats that died averaged 3093 mg./kg. or about 5 times as much as the intake of normal rats on the stock diet. This approximates the upper limits of optimal amounts of salt for maintenance of adrenalectomized rats as calculated by Anderson, Joseph and Herring (1940).

The results of these experiments in which the rats were forced to take high amounts of salt, as well as of those in which the rats voluntarily regulated their own salt intake, demonstrated that on salt therapy at least some degree of replacement can be obtained, enough to maintain food and water intake at their normal levels; they show however, that the salt therapy does not suffice to counteract the effect of what appears to be deficiency crises—during which the rats die very suddenly without any warning.

In summary 51 out of 52 adrenalectomized wild rats without nodules died despite the salt therapy, even when they received 2 to 3 times as much salt as sufficed to keep a high percentage of domesticated rats alive.

Adrenal Accessory Nodules in Wild Rats

Adrenal accessory nodules were found in 8 of 71 (11.3%) wild rats and in 3 out of 70 (4.3%) domesticated rats. The nodules were thus about 3 times as common in the wild as in the domesticated rats.

The results showed that despite the fact that adrenalectomized wild rats do not survive on salt therapy, they may thrive, even on a

"salt-poor"diet, on the secretion from a single adrenal nodule that is only a small fraction of the size of the normal gland.

One wild rat survived after the withdrawal of all therapy and in the absence of any detectable nodules. Without histological sections of the tissues in the region of the adrenals it is not possible to state with certainty that no cortical tissue was present. McFarland (1945) who made similar observations in domesticated rats, believes that after rats have been maintained for some time on salt or hormone therapy that "functional cortical masses may differentiate from coelomic mesothelium without forming all of the typical histological layers." Gaunt (1949) states that he believes that in some instances he also has seen the type of tissue described by McFarland (1945) but that he has not verified his impression with histological sections. Thus, it may be questioned whether the salt therapy kept even the one of our adrenalectomized wild rats alive.

DISCUSSION

The results of these experiments indicate that the recently trapped wild Norway rat has much greater need of adrenal secretion than does its domesticated descendant. Salt therapy in any amount does not suffice to keep the adrenalectomized wild rats alive. Additional forms of therapy are needed. How much of the adrenal cortical hormones that control electrolyte and carbohydrate metabolism is needed for full replacement has not yet been determined.

It seems likely that the captured wild rat's greater chronic tension and more violent reaction to acute stimuli accounts for its higher need for adrenal secretion. In some instances the salt therapy appeared to keep the animal alive but it did not suffice to protect it against the stress involved in reaction to acute noises in the laboratory, changes in cages and other disturbances.

During the long process of domestication the Norway rat has undergone marked anatomical, physiological and behavior changes (Richter, in press). In the present day domesticated rat, many of the other organs besides the adrenals show an atrophy. It would appear that the domesticated rat is, so to speak, a creation of the laboratory workers. For this reason the normal wild Norway may be the preferred animal for assays of the adrenal replacement therapies.

SUMMARY

Adrenalectomy had essentially the same effect in wild and domesticated Norway rats kept on "salt-poor" diets. The survival times average 8.5 days and 9.5 days respectively.

Replacement therapy with salt after adrenalectomy had very different effects in the two strains. Whereas it kept only 2% of wild Norways alive, it kept 87% of domesticated rats alive.

Their state of chronic tension and their violent reaction to acute

disturbing stimuli was the probable cause of death of the adrenalectomized wild rats.

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ALKALINE PHOSPHATASES IN VARIOUS TISSUES OF THE RAT DURING THE ALARM-REACTION

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INTRODUCTION

IN RECENT years chemical and cytochemical evidence has accumulated which indicates that hormones act by influencing enzymatic processes. The best established hormone-enzyme relationship is that which exists between acid and alkaline phosphatase and the steroid hormones. The relationship of various endocrine states to serum and prostatic phosphatase concentration was first reported by Huggins and Hodges (1941) and since extended by these investigators (Pazos and Huggins, 1945, Huggins and Russell, 1946). Serum and bone phosphatases were subsequently found to be affected by estrogen and testosterone administration (Buchwald and Hudson, 1944, 1945) as were the alkaline and acid phosphatase of the kidney, liver and intestine of the mouse and rat following castration or steroid hormone treatment (Kochakian and Flick, 1943, Kochakian and Fox, 1944, Kochakian, 1946, Tissières, 1948). The uterine alkaline phosphatase content reportedly fluctuates during the reproductive cycle in mice and in primates (Atkinson and Engel, 1947, Atkinson and Elftman, 1947). Various cytochemical studies have shown a relationship to exist between various physiological states such as pregnancy and lactation, for instance, and uterine (Wislocki and Dempsey, 1945), thyroid (Dempsey and Singer, 1946), mammary gland (Demsey, Bunting and Wislocki, 1947) and placental (Dempsey and Wislocki, 1947) phosphatase content. A recent publication by Dempsey, Greep and Deane (1949) presents the alkaline phosphatase distribution in several endocrine glands following hypophysectomy, gonadectomy and after appropriate replacement therapy. The liver content of alkaline phosphatase has been found to decrease following adrenalectomy and to markedly increase after administration of adrenalcortical extract (Kochakian, 1947).

From the preceding survey it is apparent that a close relationship exists between the tissue phosphatases and the steroid producing en-

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docrine glands. As a most rapid and intense change in steroid hormone production and general body metabolism is probably represented by the alarm-reaction, it seemed desirable to investigate alkaline phosphatase in various tissues under these conditions. We have thus studied the concentration and distribution of the alkaline phosphatase capable of dephosphorylating glycerophosphate, fructose diphosphate and yeast nucleic acid in various tissues of the rat under normal and stressed conditions.

MATERIALS AND METHODS

Thirty, male piebald rats averaging 100 gm. in weight were divided into four groups. The animals of group I served as controls. The rats comprising the other three groups were submitted to stresses of various kinds. Thus group II rats received five subcutaneous injections of 1.4 cc. of 10% formalin distributed over a 48 hours period, group III animals were exposed to cold (0–5° C.) for 48 hours while those of group IV were submitted, for the same length of time, to an atmospheric pressure sufficiently low (235–245 mm. Hg) to produce a severe anoxia. All animals were fasted throughout the experiment and killed by bleeding. At autopsy the liver, kidney, lung, spleen, lymph nodes and thymus were removed and some fixed in 95% chilled alcohol (for alkaline phosphatase) and the rest fixed in cold acetone (for acid phosphatase). Paraffin sections were cut at 5 μ and stained for alkaline and acid phosphatase using the modifications of Gomori's technique as described by Danielli (1946) Lison (1948) and Wolff, Kabat and Newman (1943). Glycerophosphate, fructose diphosphate and yeast nucleic acid were used as substrates. The solutions were buffered to pH 9.5 and 4.7 for alkaline and acid phosphatase respectively and incubated for 4 and 12 hours at 37° C. Control slides were incubated without substrate.

RESULTS

At autopsy, all of the experimental rats showed characteristic manifestations of the alarm-reaction: thymic involution, adrenal enlargement and lipid discharge, gastric ulcers (Selye, 1946) and edema of the hibernating gland (Selye and Timiras, 1949).

In most of our observations, similar results were obtained in the three experimental groups.

Alkaline Phosphatase

Liver.—After 4 and 12 hours' incubation in glycerophosphate and in fructose diphosphate, the liver of the control rats showed a picture similar to what has previously been described (Gomori, 1941, Kochakian, 1947). While only slight amounts of enzyme could be observed in the cytoplasm of the hepatic cells, their nuclei and particularly their nucleoli, were very lightly and unevenly positive. This reaction was especially localized in the periphery of the lobules. The nuclei of the Kupffer cells were positive as were those of the macrophages free in the sinusoids. The heaviest deposits occurred in the Kiernan spaces where the cells of the bile ducts and those of the peritubular

and perivascular connective tissue were sharply outlined in black (Figs. 1 and 7).

After 4 hours of incubation in yeast nucleic acid substrate, the reaction was slightly more pronounced in the nuclei of hepatic cells than with the other substrates. However, no difference could be detected in the structure of the Kiernan spaces.

The three types of stress studied produced a response identical in all experimental animals. In comparison to the controls, the enzymatic reaction in the hepatic cells was more intense and diffuse with both glycerophosphate and fructose diphosphate substrates (Figs. 2 and 8). This involved not only the nuclei and nucleoli but also the cytoplasm and particularly the cellular membrane. A similar intensification of the reaction could be detected in the Kupffer cells and in the hepatic sinusoids. No increase, or in some cases, even a slight decrease in alkaline phosphatase concentration was observed in the connective tissue and bile ducts of the Kiernan spaces.

No such increase in the alkaline phosphatase activity of the liver was observed when yeast nucleic acid was used as a substrate.

Kidney.—The location and the concentration of alkaline phosphatase in the kidney was found to be similar with the three different substrates employed. In control rats, we observed the classical distribution of alkaline phosphatase in the epithelium of the proximal convoluted tubules with a strongly positive reaction at the level of the brush border (Fig. 3). The distal convoluted tubules in the medulla and the glomeruli in the cortex were practically negative after 4 hours of incubation. When the incubation time was prolonged up to 12 hours, we observed a slight positive reaction extending to the glomeruli and the distal convoluted tubules. The adventitia of the blood vessels always stained quite markedly.

The phosphatase distribution in the kidney of the rats submitted to cold, formalin injections and anoxia did not show any detectable difference from the control animals (Fig. 4).

Lung.—Incubation of lung tissue from control rats with glycerophosphate and fructose diphosphate substrates revealed alkaline phosphatase in the nuclei of both the endothelial cells of the lung capillaries and the alveolar lining. The nuclei and cytoplasm of the bronchial epithelium were positive as well as the adventita of the pulmonary artery ramifications. The free macrophages frequently showed a strongly positive cytoplasm (Fig. 5).

With yeast nucleic acid substrate, the nuclei of the alveolar cells, the bronchial epithelium and the adventitia of arteries and veins of all sizes exhibited variable small amounts of alkaline phosphatase.

An intense increase in the alkaline phosphatase activity could be detected in all the experimental groups when using glycerophosphate and fructose diphosphate substrates (Fig. 6).

As far as the distribution and the concentration of the phosphatase

was concerned, no difference was noted between control and experimental animals with yeast nucleic acid substrate.

Spleen.—The alkaline phosphatase in the spleen of the normal animals appeared to be confined almost exclusively to the red pulp. When glycerophosphate and fructose diphosphate substrates were used, the enzyme was located in the cytoplasm of the reticular cells, the megacaryocytes and free macrophages, the content of the latter being relatively small. In the white pulp, intense phosphatase activity was demonstrable only in the endothelium of the capillaries (Fig. 11). It is interesting to note that control slides, incubated without substrate, did not show any positive reaction, excluding in this way, the possibility of the formation of iron sulphite deposits.

With yeast nucleic acid substrate, the phosphatase activity of the red pulp was less intense. The reticular cells and megacaryocytes stained only moderately.

In all the stressed animals, a marked increase in the concentration of alkaline phosphatase was noted with glycerophosphate or fructose diphosphate substrates (Fig. 12). The nuclei of the reticular cells became intensely stained as well as all macrophages. The nuclei and the nucleoli of the white pulp cells now became positive and the walls of the arterioles and capillaries showed a marked deposition of the enzyme. The control slides, incubated without substrates, were again negative.

No modification in the distribution or in the concentration of alkaline phosphatase was observed in the spleen of the experimental animals when incubated with yeast nucleic acid substrate.

Lymph Nodes.—The distribution of alkaline phosphatase in the lymph nodes exhibited great variability and the concentration was

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All microphotographs of sections incubated at 37° C. in phosphate substrates buffered to pH 9.5.

FIG. 1. Liver of a control rat incubated during 12 hours with glycerophosphate. The enzyme is located principally in the perithelial cells of the vessels and the bile ducts. Some reaction occurs also in the nuclei and particularly in the nucleoli of the hepatic cells. No reaction is observed in the cytoplasm. (Low magnification.)

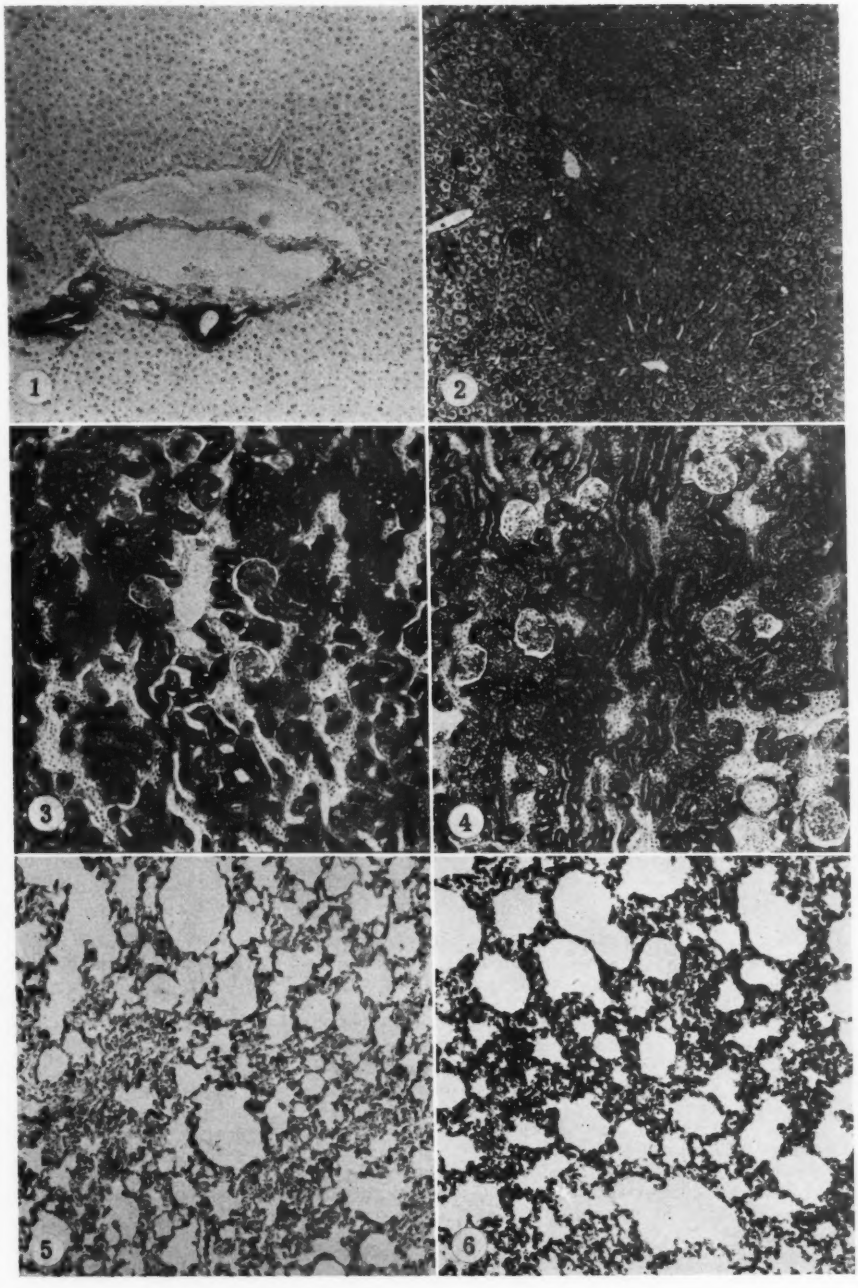
FIG. 2. Liver of a rat injected with formalin. Glycerophosphate is used as substrate. Time of incubation, 12 hours. The increase in the enzyme involves not only the nuclei and nucleoli, but also the cytoplasm of the hepatic cells. (Low magnification.)

FIG. 3. Glycerophosphatase in the kidney of a control rat. Incubation time, 12 hours. Note the classical distribution of alkaline phosphatase in the epithelium of the proximal convoluted tubules with a strongly positive reaction at the level of the brush border. The glomeruli show also a slight positive reaction. (Low magnification.)

FIG. 4. Glycerophosphatase in the kidney of a rat submitted to an atmospheric pressure sufficiently low to produce a severe anoxia. No difference can be noted from the control kidney. (Low magnification.)

FIG. 5. Lung of a control rat incubated in glycerophosphate during 12 hours. A light positive reaction is obtained in the nuclei of the endothelial cells of the capillaries and the alveolar lining. (Low magnification.)

FIG. 6. Lung of a rat injected with formalin. Glycerophosphate is used as substrate. Incubation time, 12 hours. Note the markedly increased activity of the alkaline phosphatase. (Low magnification.)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never very intense. In the lymph nodes of the control animals, when glycerophosphate and fructose diphosphate were used as substrate, the endothelium of the capillaries stained quite markedly. Some positive macrophages were also found in the medullary cords, while the germinal centers, with the exception of the capillary walls, did not show any alkaline phosphatase activity. The lymphoblasts and the lymphocytes were negative.

The capillaries and hilus arteries reacted intensely with yeast nucleic acid substrate.

In the experimental animals, there was a clear-cut increase in the amount of alkaline phosphatase. This increase was most prominent in the medullary cords and generally in the free macrophages. The lymphocytes and the cortex remained moderately colored.

Interestingly enough, with yeast nucleic acid substrate, there was no detectable increased alkaline phosphatase activity.

Thymus.—In the control group, the alkaline phosphatase activity of the thymus was the same with the three different substrates, that obtained with nucleic acid substrate being the less intense. The capillary walls were positive and, occasionally, it was possible to detect some positive macrophages (Fig. 9). In the thymocytes and reticular network the reaction was practically negative. The Hassall's bodies stained faintly. It is of interest to point out that in the thymus as well as in the lymph nodes, the granulations of the mast cells in the connective tissue surrounding the thymus and lymph nodes were deeply positive.

In the stressed animals, a greater distribution and concentration of alkaline phosphatase occurred in the cortex. In this region a relation between the intensity of the alkaline phosphatase activity and the occurrence of pycnotic centers was evident (Fig. 10). The reticular cells and the thymocytes still normal or undergoing the pycnotic process were strongly positive.

No changes were observed with yeast nucleic acid.

FIG. 7. Fructose diphosphatase reaction in the liver of a control rat. Incubation time, 12 hours. The reaction is strongest in the endothelial cells of the bile ducts. In the hepatic cells, only the nuclei and particularly the nucleoli show a slight positive reaction. (High magnification.)

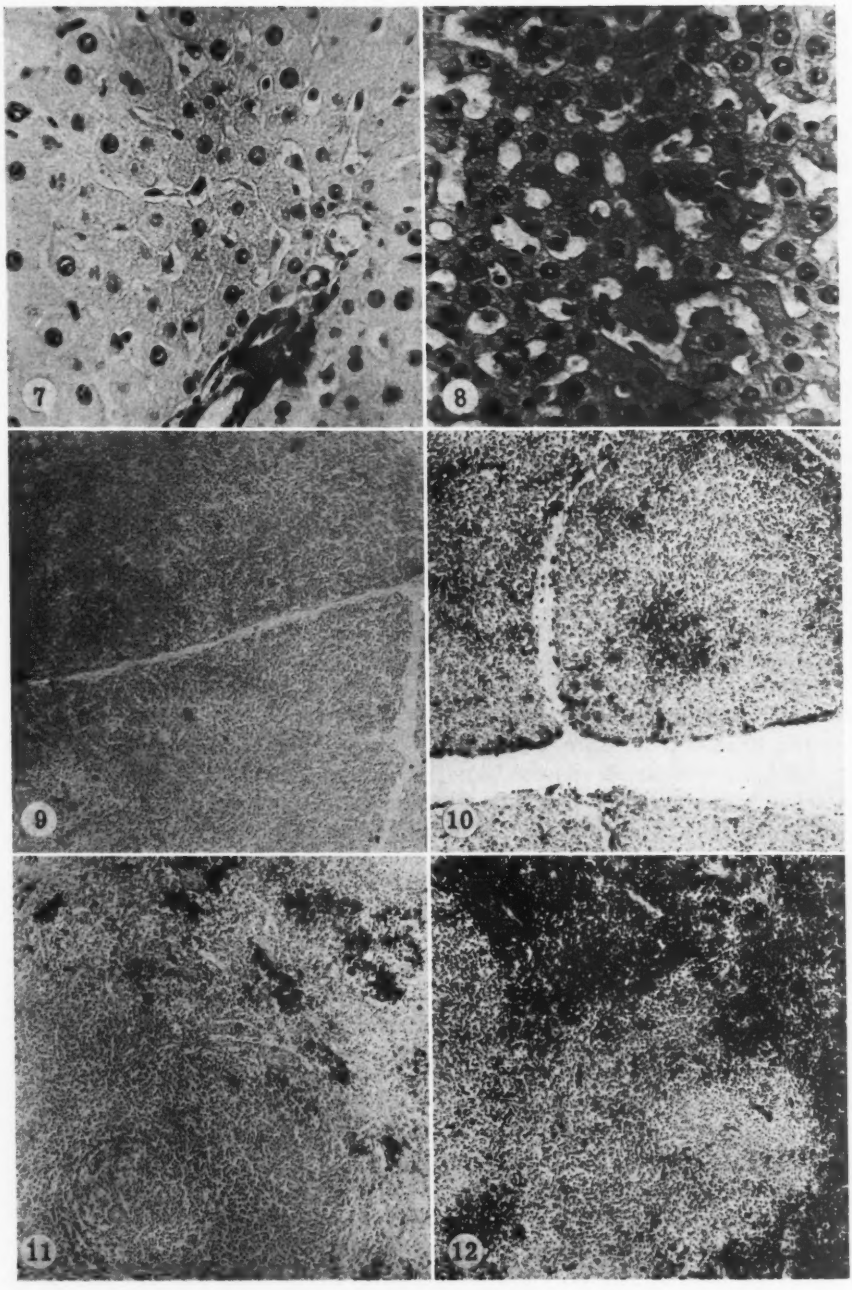
FIG. 8. Fructose diphosphatase reaction in the liver of a rat injected with formalin. Incubation time, 12 hours. The increased concentration of the enzyme is especially noted in the cytoplasm of the hepatic cells. (High magnification.)

FIG. 9. Thymus of a control rat incubated with glycerophosphate during four hours. No enzyme is present. (Low magnification.)

FIG. 10. Thymus of a rat exposed to cold and incubated with glycerophosphate during 4 hours. Strong concentration of the enzyme is seen around the area of nuclear pycnosis, especially in the cortex. (Low magnification.)

FIG. 11. Glycerophosphatase reaction in the spleen of a control rat. Incubation time, 12 hours. The enzyme appears to be confined exclusively to the red pulp. (Low magnification.)

FIG. 12. Glycerophosphatase reaction in the spleen of a rat exposed to cold and incubated with glycerophosphate substrate for 12 hours. Note the increased alkaline phosphatase activity in the red pulp. (Low magnification.)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Acid Phosphatase

Acid phosphatase was studied in a very small number of animals per group, and no final conclusions can as yet be made. However, our observations seem to indicate that there is no detectable difference in the liver content of acid phosphatase of normal or stressed rats. Further work to elucidate the variations of acid phosphatase during the alarm-reaction is at present in progress.

DISCUSSION

The results of the experiments reported here are essentially in agreement with the prevailing concept of a basic relationship between the endocrine system and cellular enzymatic activity.

In the presence of a hyperactive adrenal cortex during the alarm reaction a marked increase in the alkaline phosphatase activity of the liver was observed with glycerophosphate and fructose diphosphate substrates. This finding parallels that of Kochakian, who observed, both chemically and histochemically, a similar increase of this enzyme in the liver following corticoid injections (1944, 1947). We considered that these observations can support the hypothesis that alkaline phosphatase plays a rôle in the metabolism of carbohydrate and proteins.

It is generally agreed that a phosphorylated intermediary such as the Cori ester is a necessary antecedent to glycogen formation which then proceeds by dephosphorylation of the hexose phosphate with condensation into glycogen. At this stage, for the transformation of the hexose phosphate into glycogen, the presence of a phosphate splitting enzyme is required and, of the various enzymes capable of such action, the alkaline phosphatase has been most frequently invoked. However, there is a definite difference between the effect of corticoid administration and the increased corticoid production from the adrenal during the alarm-reaction. In both cases, the metabolism of carbohydrate seems to be affected, but, while corticoid administration increases the deposition of glycogen in the liver during the alarm reaction, peripheral metabolism probably accelerates the immediate utilization of any newly formed glycogen. Hence, during the alarm-reaction the alkaline phsophatase could be related not only to the process of glycogenesis but also to glycogenolysis.

In this connection, it is necessary to remember that the various types of stress do not always affect carbohydrate metabolism in the same way. Thus, while cold seems to produce a severe and rapid exhaustion of the glycogen stores of the organism, anoxia causes an increase in glycogen deposition in the liver (Lewis, Thorn, Koepf and Dorrance, 1942; Nims, Langley and Clarke, 1946).

However, it was suggested (Kochakian, 1947) that alkaline phosphatase need not be associated only with the conversion of glucose to glycogen but may be related to the mobilization of small carbon resi-

dues obtained from amino acids. Indeed, the formation of glycogen after administration of carbohydrate is not accompanied by an increase in alkaline phosphatase, while feeding a high protein diet does cause such an increase.

Of more difficult interpretation seem to be the heavy deposits of alkaline phosphatase which occur in the liver at the level of the bile ducts, the sinusoids and the Kupffer cells. Similarly surprising is the lack of increase in alkaline phosphatase when yeast nucleic acid was used as a substrate.

In agreement with the earlier results of Kochakian obtained by the injection of corticoids in normal and adrenalectomized rats, no difference between the alkaline phosphatase activity in the kidney of control and experimental animals could be detected, with any substrate employed.

Little is known of the rôle of the lung during the alarm reaction. Recent experiments performed in this laboratory (Timiras and Selye, 1949) have shown that, after exposure to stress, there is an increased phagocytosis of India ink particles by the reticulo-endothelial system and that this reaction is particularly marked in the lung. Perhaps the increased activity of alkaline phosphatase in this organ during the alarm reaction could be related to some unknown mechanism of defense.

In the spleen the normal, positive reaction of variable intensity in the cells of the reticular network and free macrophages of the red pulp becomes more generalized and the phosphatase concentration increases very markedly during the alarm reaction. The contraction of the spleen which occurs after exposure to alarming stimuli must be considered when reviewing the possible causes of this increase in the alkaline phosphatase activity because of the increase in density of the red pulp. However, at high magnification, all the reticular cells showed a very clear-cut positive reaction in the stressed animals. The white pulp, on the other hand, reacts like the lymph nodes and thymus with a marked increase in the enzymatic activity, which, however, remains quite irregular. A few lymphoblasts and lymphocytes and the reticular cells stained quite lightly.

In the lymph nodes, the increase in the alkaline phosphatase activity during the alarm-reaction is especially marked in the reticular cells of the medullary cords, in the free macrophages and even in some lymphoblasts and lymphocytes. It is much more difficult in the lymph nodes to detect a relationship between the caryoclastic phenomenon and the increase in the phosphatase activity which can be observed very easily in the thymus. The alkaline phosphatase activity of the thymus is particularly evident in the cortex around the caryoclastic foci. The fact that there are marked differences with two of the substrates employed—glycerophosphate and yeast nucleic acid—seems to suggest that the caryoclastic phenomenon, especially in the thymus, is related to the action of corticoids on carbohydrate metabolism.

SUMMARY AND CONCLUSIONS

Alkaline phosphatase distribution and concentration were studied in normal rats and in rats exposed to alarming stimuli (formalin, cold and anoxia). When using glycerophosphate and fructose diphosphate as substrates, a marked increase in the phosphatase activity could be detected in the liver, lung, spleen, lymph nodes and thymus. No such increase was observed in these organs with yeast nucleic acid substrate. In the kidney, no difference was noted between the alkaline phosphatase of the control and of the experimental animals, whatever substrate was employed.

The rise in the activity of the liver and lymphatic tissue alkaline phosphatase appears to parallel the increased metabolism of these tissues during exposure to stress. Likewise, as there is a similar parallelism between the increased intensity tissue phosphatases and endocrine activity, the observations presented here seem to indicate a close relationship between this enzyme and corticoid secretion during the alarm-reaction.

ACKNOWLEDGMENTS

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EFFECTS OF STILBESTROL ON WEIGHT AND ASCORBIC ACID LEVEL OF GUINEA PIG ADRENALS

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INTRODUCTION

DURING the course of a study of the histogenesis of the estrogen-induced tumors of guinea pigs (Nadel, 1949), the influence of ascorbic acid upon the development of this type of tumor was investigated. An unexpected finding, compatible with acute scurvy (Meyer and McCormick, 1928), developed when stilbestrol pellets were implanted into guinea pigs kept on a diet partially deficient in ascorbic acid.

A study of the possible interrelationship between ascorbic acid and stilbestrol metabolism was undertaken. This paper deals with effects of stilbestrol implants on adrenal ascorbic acid concentration and adrenal weights of female guinea pigs maintained at different levels of ascorbic acid deficiency.

METHODS

Stock virgin female guinea pigs raised at the National Institutes of Health Breeding Farm were used in all the experiments. A stock diet of commercial Purina rabbit chow and fresh cabbage was supplemented with carrots once a week. Animals were transferred from this stock diet to the scorbutogenic diet described below. The day of transfer is indicated as Day 0.

Scorbutogenic diet: A special diet, prepared in pellet form from a formula by Morris (1947) is given below. When guinea pigs weighing between 275–325 grams were transferred to this diet, they died with clinical signs and histological evidence of scurvy within 35 days.

Morris Scorbutogenic Diet

	Pounds
Alfalfa (ground)	900
Whole wheat (ground)	336
Molasses	150
Ground oats	100
Soy bean oil meal	100
Cerogras	100
Peanut oil meal	100

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Wheat bran	80
Rice offal	50
Salt (NaCl)	40
Apple pomace	30
Wheat germ	20
Brewer's dehydrated yeast	10
Shark oil	2
Liver meal	2
Mannamar	2
Irradiated yeast	1

Pellets, thus prepared, were mixed with shark and wheat germ oils just before use to insure against the loss of vitamins through storage. Twenty grams of each of the oils were added dropwise to 10 pounds of diet and the feed stirred slowly by hand over a period of 20–30 minutes to insure a good distribution of the added factors. Twenty pounds of feed were prepared in this manner twice weekly and kept at 4° C. Directly before each feeding, an additional supplement in the form of 10 grams of powdered Brewer's yeast was dusted onto each 1000 grams of diet after the method of Reid (1947, a).

Ascorbic acid solutions were prepared by dissolving crystalline ascorbic acid in distilled water and adjusting the resultant solution to pH 6.9 with 0.5% sodium bicarbonate. From a stock solution of 100 mg. of ascorbic acid per ml., dilutions were prepared for intraperitoneal injection immediately before use. Aliquots of these solutions analyzed immediately after injections showed no loss in ascorbic acid.

Hormone pellets: Pellets of 40% stilbestrol in cholesterol and of 100% cholesterol were prepared by fusion after established methods (Shimkin and White, 1941). The weights of the pellets thus prepared ranged from 35–45 mg. They were inserted intramuscularly in the dorsal cervical midline through a small surgical incision which was closed by a single Michel clip. All pellets were removed and weighed at the time of autopsy. Losses in the weight of these pellets, presumably because of absorption by the tissues of the guinea pigs, averaged less than 2 mg.

Post-mortem examinations: Animals were sacrificed by a blow on the head. The adrenal glands were removed quickly, placed in a Petri-dish surrounded by dry ice until partly frozen and then weighed to the nearest mg. on a Roller-Smith micro torsion balance. Individual glands were kept in dry ice until analyzed for ascorbic acid. The analyses were made usually within 15 minutes after the removal of the gland.

Ascorbic acid analysis: The adrenals, frozen in solid carbon dioxide, were homogenized for 1 minute in a Semimicro Waring Blendor with 50 ml. of ice-cold metaphosphoric-acetic acid mixture. Homogenate thus prepared was mixed with celite and filtered. The ascorbic acid was determined on aliquots of filtrates by the titrimetric method of Bessey (1944).

Experimental procedures: All animals were maintained on the stock diet prior to transfer to the special experimental diet. Twenty-seven animals on the stock diet (Series 1) were sacrificed at intervals to determine the normal relation of adrenal weight to body weight and adrenal ascorbic acid concentration (Figure 1).

In Series 2, twenty-six animals were placed on the scorbutogenic diet. These were serially sacrificed at regular intervals to study the effects of ascorbic acid deficiency on the adrenal ascorbic acid concentration (Figure 2).

TABLE 1. EFFECT OF STILBESTROL ON THE WEIGHT AND ASCORBIC ACID CONCENTRATION OF THE ADRENALS OF GUINEA PIGS ON THE SCORBUTOGENIC DIET

Group	Treatment		Adrenal wt. (mg.)	AD/W	Adrenal ascorbic acid (mg. %)
	Stilbestrol	Ascorbic acid[1] (mg./day)			
W	+	0	275	101	11
			294	110	10
			253	107	13
Average ± S.D.			274	106 ± 2.65	11.3 ± 0.9
X	0	0	159	73	16
			160	74	25
			204	82	18
Average ± S.D.			174	76.3 ± 2.86	19.7 ± 2.7
Y	0	25	155	67	95
			156	58	108
			154	63	100
			155	52	94
Average ± S.D.			155	60 ± 3.24	99.2 ± 3.2
Z	+	25	311	107	31
			208	76	61
			211	84	40
			210	77	25
Average ± S.D.			235	86 ± 7.23	39.2 ± 7.9

[1] Groups W and X were sacrificed on Day 23. Groups Y and Z were injected intraperitoneally with 25 mg. ascorbic acid on Days 24 and 25 only and were sacrificed 18 hours after the second dose.

In Series 3, a study was made of the effect of stilbestrol on the weight and ascorbic acid content of the adrenal glands of guinea pigs on a scorbutogenic diet as well as during recovery subsequent to ascorbic acid therapy (Table 1). Fourteen animals (250–300 gm.) were divided into groups W, X, Y and Z. Groups W and Z received stilbestrol implants on Day O while X and Y received control cholesterol blanks. All groups were kept on the scorbutogenic diet for 23 days when groups W and X were sacrificed. The animals in groups Y and Z received a daily intraperitoneal injection of 25 mg. of ascorbic acid on Days 24 and 25 only, and were sacrificed 18 hours after the second ascorbic acid injection.

In Series 4, a study was made of the effects of stilbestrol and varying amounts of ascorbic acid on the adrenal weight and on the adrenal ascorbic acid concentration of guinea pigs on the scorbutogenic diet (Table 2). Twenty-eight animals placed on the scorbutogenic diet were divided into groups A, B, C, D, and E. On Day O groups A and B were treated with stilbestrol implants while groups C, D, and E received cholesterol pellets. Daily intraperitoneal ascorbic acid injections of 4 mg. (a scurvy preventive dose) each were given for 14 days to animals in groups A and C, and 100 mg. (a high dose) to animals in groups B and D. Thus animals in groups A and B received stilbestrol plus 4 and 100 mg. of ascorbic acid daily, respectively; those in groups C, D, and E had 4, 100 and 0 mg. of ascorbic acid daily,

TABLE 2. EFFECTS OF STILBESTROL AND ASCORBIC ACID ON ADRENAL ASCORBIC ACID AND AD/W[1] OF GUINEA PIGS ON SCORBUTOGENIC DIET

Group	Treatment		Adrenal wt. (mg.)	AD/W	Adrenal ascorbic acid (mg. %)
	Stilbestrol	Ascorbic Acid[2] (mg./day)			
A	+	4	206	62	88
			228	74	38
			268	90	24
			270	111	16
			182	59	55
Average±S.D.			231	79.3±9.64	44.2±13.
B	+	100	208	68	121
			165	52	106
			224	75	112
			212	76	118
			216	79	156
			136	50	114
			152	57	129
Average±S.D.			188	65.3±4.58	122.3±6.
C	0	4	94	38	91
			106	40	122
			150	56	89
			120	44	96
			102	36	57
Average±S.D.			114	42.8±3.59	91±10.
D	0	100	128	42	140
			90	31	156
			91	31	173
			111	40	158
			130	49	121
Average±S.D.			110	38.6±3.45	149.6±9.
E	0	0	102	43	35
			116	53	31
			114	57	19
			132	46	28
			106	45	34
			117	52	29
Average±S.D.			115	49.3±2.4	29.3±2.

[1] AD/W = mg. adrenal weight per 100 gm. body weight.
[2] Ascorbic acid was injected intraperitoneally once daily for 14 days.

respectively, and no stilbestrol. All groups were sacrificed at the end of the experimental period which was 18 hours after groups A through D had received their 14th (last) daily injection of ascorbic acid.

RESULTS AND DISCUSSION

Relation of adrenal weight to body weight is graphically shown in Figure 1 where adrenal weight in milligrams per 100 grams of body weight (AD/W) is plotted against body weight. Adrenal weight increased at a faster rate than body weight. As a result AD/W for

growing animals was greater with increase in age and weight. Animals weighing 150–350, 400–500 and 501–800 gm. had mean AD/W of 42.9, 52.4 and 68.8, respectively. Mixner, Bergman and Turner (1943) reported similar findings.

Adrenal ascorbic acid concentrations determined on 27 growing animals on regular stock diet averaged 144 ± 5 mg. %. Individual ascorbic acid values ranged from 103–241 mg. %. The adrenal ascorbic acid concentration was 110–120 mg. % for five animals, 121–140 for eight, 141–160 for seven, 161–180 for five, 103 for one, and 241 for one. These adrenal ascorbic acid values do not show any correlation

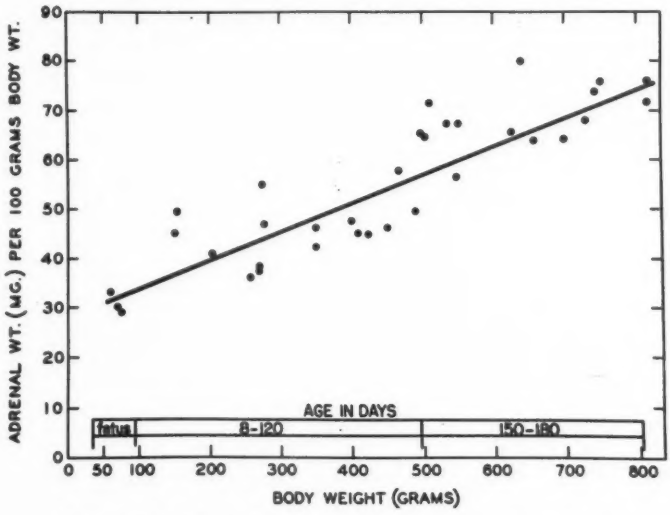

FIG. 1. Relationship between adrenal weight (in mg. per 100 gm. of body weight) and body weight.

with the age of the animal, its body weight or the weight of the adrenal glands. Reid (1947, b) has observed a wide range in the adrenal ascorbic acid values of male guinea pigs. Normal values for individual animals as reported in the literature range from 75 mg. % (Follis, 1947) to 336 mg. % (Roe, Hall, and Dyer, 1941). The average value reported by Kuether, Telford, and Roe (1944) was 118 mg. % and that reported by Long (1947, a) was 137 mg. %.

The effect of the scorbutogenic diet on the adrenal ascorbic acid concentration is graphically illustrated in Figure 2. The mean adrenal ascorbic acid values with standard errors obtained from guinea pigs serially sacrificed are plotted against the time during which the animals were on the scorbutogenic diet. A free hand curve was drawn through the mean values for Day 0 and for each day on the scorbutogenic diet. With the Day 0 mean adrenal ascorbic acid value of

144 mg. %, those animals on the scorbutogenic diet for 1, 4, 14, 23, and 33 days show a drop of 32, 70, 80, 88, and 95%, respectively, in adrenal ascorbic acid concentrations.

Our animals lost about 32% of their adrenal ascorbic acid concentration when kept on the scorbutogenic diet for 24 hours, while Venning, Kazmin and Bell (1946) and Long (1947, b) found that their animals, which had been getting adrenocorticotropic hormone injections, lost only 10–15% under otherwise similar conditions of ascorbic acid starvation. Has adrenocorticotropic hormone a sparing effect on adrenal ascorbic acid depletion? The mechanism of adrenal ascorbic

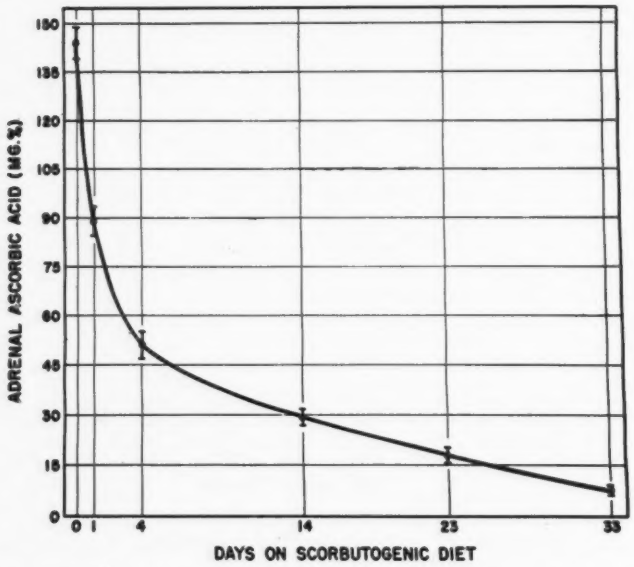

FIG. 2. Depletion of adrenal ascorbic acid in guinea pigs on scorbutogenic diet.

acid depletion and its replenishment needs further study for its proper understanding.

Changes in AD/W and adrenal ascorbic acid concentration caused by the scorbutogenic diet, stilbestrol therapy and the subsequent degree of recovery after ascorbic acid injections are shown in Table 1. Scorbutogenic diet alone (group X) increased the AD/W from about 40, normal for 200–300 gm. animals, to about 75. This value was further increased to about 105 in stilbestrol-treated animals (group W). Adrenal ascorbic acid concentrations in stilbestrol-treated animals were significantly lower than those in non-treated cases. When ascorbic acid was administered to animals of groups Y and Z, those without stilbestrol (group Y) showed a remarkable rise in their adrenal ascor-

bic acid values to low normal, while in the stilbestrol-treated group Z, the rise in this value was comparatively small. The degree of recovery of AD/W toward normal was similar both in stilbestrol-treated (groups W vs. Z, 19%) and non-stilbestrol treated animals (groups X vs. Y, 21%). Thus treatment with stilbestrol decreased the adrenal ascorbic acid concentration over and above its depletion due to the scorbutogenic diet and these treated animals did not respond to ascorbic acid therapy as readily as ones without stilbestrol. The presence of stilbestrol in animals appeared to prevent the return of the adrenal ascorbic acid concentration to normal levels within the time studied.

Effects of stilbestrol and known ascorbic acid therapy on animals given the scorbutogenic diet are recorded in Table 2. In this series, 200–300 gm. animals were placed on the scorbutogenic diet for 2 weeks to avoid the weight losses reported when guinea pigs are kept on scurvy-producing diets longer than 15 days (Dunker, Fellers and Esslen, 1942). All but 3 of the animals gained weight.

Stilbestrol-treated animals, groups A and B, had the largest AD/W. Animals receiving 100 mg. of ascorbic acid daily (group B) were in the low normal range for adrenal ascorbic acid (122 mg.%), while those receiving 4 mg. daily (group A) had a value of 48 mg.%.

The daily administration of 4 mg. of ascorbic acid to groups A and C was calculated to provide the dose (1.23 mg.% of body weight) which Kuether, Telford and Roe, (1944) reported as sufficient to prevent changes associated with scurvy in the teeth of guinea pigs. Comparison of animals receiving the scurvy preventive dose of 4 mg. of ascorbic acid, group C, with those receiving 100 mg., group D, shows that the adrenal ascorbic acid concentration is much higher and the AD/W slightly lower in the latter group.

Animals receiving 4, 100, and 0 mg. of ascorbic acid and no stilbestrol, groups C, D, and E, respectively, had lower values for AD/W than groups A and B which received stilbestrol. The AD/W for control group E was slightly higher than that for groups C and D. Adrenal ascorbic acid concentration in these groups increased with increase in ascorbic acid intake.

SUMMARY AND CONCLUSIONS

Adrenal ascorbic acid concentration of 144 ± 5 mg.% has been established as the mean normal value for growing female guinea pigs used in these experiments.

Scorbutogenic diet reduced this mean normal value of 144 mg.% to 90 mg.% in 24 hours, a 32% reduction, and to 7.6 mg.% in 33 days, a 95% reduction.

The adrenal weight of normal growing animals increased at a much faster rate than body weight.

Ascorbic acid deficiency was associated with an increase in adrenal

weight and AD/W (mg. adrenal weight per 100 gm. body weight) in guinea pigs.

Estrogen-treated animals always had higher AD/W and lower ascorbic acid concentration than their non-treated counterparts, regardless of ascorbic acid intake of the animals. Increase in ascorbic acid intake increased the adrenal ascorbic acid concentration.

Estrogens induced enlargement of the adrenal gland and caused an increase in AD/W. Increase in AD/W in growing animals might be a reflection of the normal increase in estrogens as the animals grow older.

Ascorbic acid concentration in the adrenal gland of normal growing guinea pigs seemed to be a resultant of ascorbic acid intake and rate of weight increase of the adrenal gland with age. There might be dietary and other factors involved in determining the ability of the adrenal gland to concentrate available ascorbic acid.

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